IMPACT OF PROTEIN-FREE MEDIUM, DURATION OF INCUBATION AND TEMPERATURE ON SPERMATOZOA ACTIVITY POST- PREPARATION

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Field of Study: **HUMAN INFERTILITY**

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ABSTRACT

The objectives of this study were to identify factors that could retain the activity of human spermatozoa over extended periods of time, and to establish the efficacy of the synthetic chemically defined protein-free spermatozoa handling and culture media on spermatozoa motility and vitality. Semen samples were obtained following informed consent. In Experiment 1 semen samples were prepared by density gradient centrifugation. The resulting re-suspended pellet was apportioned equally in the commercial protein-containing medium (CPC); minimum essential medium + HSA (MEM) or the synthetic protein-free medium (PFM). In Experiment 2 the motility of spermatozoa held for 24hrs at 4ºC, 15ºC, 22ºC or 37ºC in CPC, MEM or PFM media were determined. In Experiment 3 the vitality and viability of human spermatozoa held at room temperature were evaluated at 0hr, 4-7hrs and 24hrs post-wash. There is no difference in the activity of human spermatozoa in the protein-containing or protein-free handling and culture media. There is no significant difference in the spermatozoa motility held at different temperatures for 4-7hrs post-wash, but it was highest at 22ºC; likewise after 24hrs the significantly highest motility was noted at 22ºC than 37ºC, 15ºC and 4ºC ($p<0.05$) in all media. The same was true for spermatozoa vitality and membrane integrity at 4-7hrs, but a significant reduction was noted after 24hrs at 37ºC ($p<0.05$). There were no significant changes in the DNA integrity levels when spermatozoa was held at 22ºC or 37ºC for 4-7hrs or 24hrs in all media. In conclusion the activity of spermatozoa can be successfully retained at room temperature for up to 24hrs without major detriment. The efficacy of the protein-free media is equally efficient as the conventional protein-containing media.

ABSTRAK

Objektif kajian ini adalah untuk mengenalpasti faktor faktor yang boleh mengekalkan aktiviti sperma dalam tempoh masa tertentu, menentukan keberkesanan media sintetik tanpa-protin ke-atas sperma, dan menentukan pengaruh suhu keatas aktiviti sperma Sampel air mani diperolehi dengan izin pesakit. Dalam eksperiment 1, sampel disediakan dengan cara ketumpatan putaran densiti dan larutan semula pelet yang dibahagikan sama rata ke dalam media komersial yang mangandungi protin (CPC); media penting minima+serum albumin manusia (MEM) atau media sintetik tanpa-protin (PFM). Manakala, di dalam eksperimen 2, sperma didedahkan pada suhu 4°C, 15°C, 22°C atau 37°C dalam CPC, MEM atau PFM media untuk tempoh masa 24 jam. Tiada perbezaan yang nyata pada aktiviti spermatozoa di dalam CPC/PC atau PF media. Dalam semua media, tiada perbezaan yang nyata dapat direkodkan pada pergerakan sperma untuk 4-7 jam selepas cucian, tetapi pergerakan paling laju adalah pada suhu 22^{o}C; sementara selepas 24 jam, pergerakan sperma yang paling laju juga adalah pada 22^{o}C berbanding 37^{o}C, 15^{o}C dan 4^{o}C (p<0.05) dalam semua media. Persamaan ini dapat dilihat dengan jelas untuk daya hidup sperma dan integriti membran pada 4-7 jam, tetapi penurunan yang nyata dapat direkodkan selepas 24 jam pada suhu 37^{o}C (p<0.05). Tiada perubahan yang nyata dalam tahap integriti DNA apabila sperma diletakkan pada suhu 22^{o}C atau 37^{o}C untuk 4-7 jam atau 24 jam dalam semua media. Ini dapat disimpulkan bahawa aktiviti sperma dapat dikekalkan dengan jayanya di dalam suhu bilik sehingga 24 jam. Keberkesanan media sintetik tanpa-protin adalah sama efisien seperti media koventional yang mengandungi protin.

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LIST OF SYMBOLS AND ABBREVIATIONS

ART	:	Assisted Reproductive Technology
cIVF	:	conventional *In Vitro* Fertilization
CO_2	:	Carbon dioxide
CPC	:	Commercial Protein-Containing Medium
DFI	:	DNA fragmentation index
DGC	:	Density Gradient Centrifugation
DNA	:	Deoxyribonucleic Acid
DSPs	:	Donor Serum Proteins
ESHRE	:	European Society for Human Reproduction and Embryology
Expt	:	Experiment
H_2O	:	Dihydrogen Oxide
HA	:	Human Albumin
hCG	:	Human chorionic gonadotropin
HFEA	:	Human Fertilization and Embryology Authority
HKL	:	Hospital Kuala Lumpur
HOT	:	Hypo-Osmotic Test
HSA	:	Human Serum Albumin
ICSI	:	Intracytoplasmic Spermatozoa Injection
IRB	:	Institutional Review Board
IUI	:	Intrauterine Insemination
IVF	:	*In Vitro* Fertilization
JA	:	Jaffar Ali
LPPKN	:	Lembaga Penduduk dan Pembangunan Keluarga Negara
MEM	:	Minimum Essential Medium
OPU	:	Ovum Pick Up

PFM	:	Synthetic Protein-Free Medium
rHA	:	recombinant Human Albumin
ROS	:	Reactive Oxygen Species
RT	:	Room Temperature
SART	:	The Society for Assisted Reproductive Technologies
SDF	:	Spermatozoa DNA fragmentation
SVS	:	Vital Stain solution
UMFC	:	University of Malaya Fertility Centre
UMMC	:	University of Malaya Medical Centre
WHO	:	World Health Organization
ZP	:	Zona Pellucida

LIST OF MEASURMENT UNITS

%	:	Percentage

hrs	:	Hours
°C	:	Degrees Celcius
min	:	Minute
mOsm	:	MilliOsmols
mg/ml	:	Milligram per milliliter
ml	:	Milliliter
µl	:	Microliter
sec	:	Second
rpm	:	Revolutions per minute
g	:	Gram

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Infertility, particularly male infertility, is a matter of major concern, as it is a clinical challenge of increasing significance, especially for couples yet to achieve parenthood. The World Health Organization (WHO), defines infertility as a condition where a couple is unable to conceive spontaneously despite unprotected, regular sexual intercourse for more than one year (Rowe *et al.,* 2000). Globally, an estimation of 50-80×10^6 couples are infertile (Rutstein & Shah, 2004). In a recent statistics by the European Society for Human Reproduction and Embryology (ESHRE, 2014), reported that one in six couples worldwide experience some form of infertility or subfertility, at least once during their reproductive lifetime. The current prevalence of infertility is estimated to be around 9% worldwide for women of reproductive age 20-44 (ESHRE, 2014). Therefore, many childless couples seek medical assistance to conceive.

Assisted Reproductive Technology (ART), is group of treatment modalities used to achieve pregnancy by unnatural means using procedures that involve fertility medication, artificial insemination and *in vitro* fertilization (IVF) techniques. The birth of Louise Brown, the world's first test tube baby on July 25th, 1978 through the pioneering work of Dr Robert Edwards and Dr Patrick Steptoe heralded much hope for the childless couples. The European Society for Human Reproduction and Embryology (2014) reported that "more than five million babies born using ART worldwide" since the first IVF baby was born in 1978.

The ART treatment modality includes the conventional *in vitro* fertilization (cIVF), Intracytoplasmic Spermatozoa Injection (ICSI) and Intrauterine Injection (IUI). In cIVF surgically removed eggs are fertilized *in vitro* by co-incubating spermatozoa with

the egg to enable penetration by a single spermatozoon and fertilization of the egg by natural mechanisms. The European IVF pregnancy rate in 2011 per embryo transfer was 33.2% (ESHRE, 2014). The Society for Assisted Reproductive Technologies of the United States of America (SART), reported a pregnancy rate 46.7% (<35 years old), 37.8% (35-37 years old), 29.7% (38-40 years old), 19.8% (41-42 years old) and 8.6% (>42 years old) following IVF. The intracytoplasmic spermatozoa injection (ICSI) modality is one the most frequently performed ART treatment. ICSI is achieved when a single spermatozoon is injected directly into an egg; this is often used for couples with male factor infertility. The pregnancy rate per ICSI cycle per age group is about 39.1% (<35 years old), 36.6% (36-39 years old) and 17.1% (<40years old) as stated by the Human Fertilization and Embryology Authority (HFEA), United Kingdom (2009).

Meanwhile, the intrauterine insemination (IUI) is the cheapest and most common ART treatment for the unexplained infertility. It is achieved when the washed spermatozoa is injected into the uterus using a catheter near to the egg and close to the time of ovulation. Although IUI is less invasive than IVF and ICSI, the success rates have varied widely. Unfortunately, over the last 30 years the IUI pregnancy rates have remained stagnant around 10-15% following 4-6 well timed cycles of insemination (Veltman-Verhulst *et al.*, 2012).

Most of the assisted reproductive treatments require a selection of washed specimen from the ejaculated spermatozoa before the performance of the treatment. This is an essential prerequisite step in the ART treatment procedures, primarily because the semen is toxic (Bjorndahl *et al.*, 2005; Dott, 1974; Shannon, 1965a, b). Human ejaculation comprises of a mixture of seminal plasma, mature and immature spermatozoa, non-reproductive cells, various microorganisms as well as non-specific debris (Zinaman *et al.*, 2000). However, some components of the seminal fluid may become an obstacle when *in vitro* fertilization is performed (Bjorndahl *et al.*, 2005). Spermatozoa and leukocytes in

the semen sample produce many oxygen radicals that alter the possibility of the spermatozoa-oocyte fusion (Natali, 2011). In addition, the seminal fluid also contains various gamete-toxic and embryo-toxic factors such as free radicals, cellular debris and dead spermatozoa (Bjorndahl *et al.,* 2005). Therefore, the motile fraction of spermatozoa must be isolated from these toxic components and kept intact until the right time of insemination to enable an effective ART treatment procedure. Due to the important role of spermatozoa washing and preparation steps a number of techniques have been described (reviewed by Ali, 2014).

Several spermatozoa separation methods are now available, for example, the direct washing technique, swim up, semen filtration and the density gradient centrifugation (DGC) technique (Henkel & Schill, 2003), which must be carried out as a means to select a greater number of motile, live and normal morphology spermatozoa (Morrell *et al.,* 2009). All these techniques are capable of effectively separating the spermatozoa from the seminal plasma, but to varying degree. Recovery rates, motility, morphology and the degree of DNA damage vary greatly between procedures (*Dicky et al.,* 1999). In addition, some adverse effects to the spermatozoa motility, mitochondrial function, viability rate, DNA integrity level, spermatozoa surface receptors and on some gene expression have been reported (Zini *et al.,* 1999& 2000). Therefore, the need to improve spermatozoa processing and culturing of actively motile and viable spermatozoa has increased tremendously (Noah *et al.,* 2004). Spermatozoa preparation techniques have evolved over the years; with the advancement in the techniques of the assisted reproduction procedures, an increase in the knowledge of spermatozoa physiology and function as well as the understanding of genetic contribution to fertilization rate and embryo development. In this respect, there has been a rising concern over the safety of the spermatozoa separation procedures and the washing or preparation media or reagents with respect to not only the motility of the harvested spermatozoa, but also on the

viability, DNA integrity and to the long-term effects on the resulting pregnancy (Mussart & Kalsoom, 1998).

Spermatozoa quality is a very important factor in the IVF outcome, since the male infertility accounts for 30-50% of the infertility cases and the treatment options are mainly based on the spermatozoa quality (Elder & Dale, 2011). The fertilization of an oocyte with an apoptotic spermatozoon has been shown to have detrimental effects on the fertilization and implantation rate as well as on the embryo development in the assisted reproduction treatment (Vantery Arrighi *et al.,* 2009). Additionally, the occurrence of the acrosome reaction is also essential to achieve fertilization (Grunewald *et al.,* 2006).

In general, it is established that spermatozoa quality is important in maintaining the reproductive potential of men (Agarwal & Allamaneni, 2004). Besides, the fertilizing potential of the spermatozoa depends not only on the functional competence of the harvested spermatozoa for insemination, but also on other genomic components and factors present in the semen sample (Saleh *et al.,* 2003; Sharma *et al.,* 2004). Spermatozoa function assessments could also provide a valuable indication of spermatozoa quality and its fertility potential.

Classical semen analysis, which express the spermatozoa concentration, motility, viability and morphology (Ombelet *et al.,* 1997a; WHO, 2010) gives an approximate evaluation of the functional competence of the spermatozoa, but does not detect spermatozoa abnormalities in about 20% of infertile men (Romeo *et al.,* 2001). Men with spermatozoa of normal morphology may still be infertile; the cause could be related to abnormal spermatozoa DNA integrity (Agarwal & Allamaneni, 2004; Alvarez *et al.,* 2003), mitochondrial function, spermatozoa surface receptors, vitality, plasma membrane integrity level as well as the DNA gene repair expression, which have an important role

not only for fertilization but also for normal embryo implantation and foetal development (Morris *et al.*, 2002).

Donor serum protein is routinely added to the culture and handling media (Freshney, 1987), which mainly helps to prevent the spermatozoa from sticking to each other or to the plastic ware, and also to provide nitrogenous substrate for the cellular metabolism. The proteins used for human ART program are in the form of the patient's serum, bovine serum albumin or various human serum fractions. However, the use of blood or blood-based products can be risky, as patients have been infected with hepatitis after undergoing transfusion of donated blood (Holmes, 1989). The constant emergence of new viruses and other infective agents makes the risk of infection through the donated serum in the media a perennial problem.

Many studies and investigations have been carried out to find a source that can replace the use of the donor serum protein in the culturing and handling media, for example, non-animal macromolecules, synthetic and plant molecules, recombinant human albumin (rHA) and the protein-free media (Ali *et al.,* 2000; Bavister *et al.,* 2003; Gardner & Lane, 1999; Leung *et al.,* 1984; Parinaud, *et al.,* 1999 & 1998).

1.2 PROBLEM STATEMENT

Human spermatozoa preparation protocol may affect some features of the spermatozoa, as it may disturb its quality and DNA integrity level. During the semen sample processing the spermatozoa will be exposed to some environmental insults, as it is placed in different washing and culturing media. This may result in major changes in the temperature, pressure, light intensity, acidity and the reactive oxygen species (ROS) levels, which may subsequently result in an alteration in the fertilization potential of the harvested spermatozoa which as a consequence could culminate in poor outcome.

Increased levels of ROS exhibit significantly elevated levels of DNA damage (Aitken *et al.,* 1998). There is also evidence that spermatozoa DNA integrity is associated with lower natural pregnancy rates (Loft *et al.,* 2003; Spano *et al.,* 2000) and lower IUI/IVF/ICSI outcomes (Evenson *et al.,* 2008).

Spermatozoa with compromised DNA integrity, regardless of the degree of DNA damage, appear to have the capacity to fertilize oocytes at the same rate as the normal spermatozoa do (Agarwal & Allamaneni, 2004). However, embryo development can be affected depending on the degree of the DNA damage, thus it may result in embryonic death (Seli *et al.,* 2004). In addition, the fertilization of oocytes by ICSI with DNA damaged spermatozoa may have serious consequences for the progeny if the damage is transferred to the germ line. In this respect, increased chromosomal abnormalities, minor or major birth defects or childhood cancers have been reported (Aitken & Sawyer, 2003; In't Veld *et al.,* 1995). Data on hundreds of semen samples (Singh *et al.,* 2003) show that patients with a DNA fragmentation level of greater than 30% are likely to have significantly reduced fertility potential as well as a greater risk of miscarriage.

1.3 SIGNIFICANCE OF THE STUDY

Advancement in human ART over the last 30 years has made these techniques accessible to the infertile couple in almost every corner of the globe and have raised the possibility for childless couples to achieve pregnancies. This current investigation is a prospective study, which has been approved by the Medical Ethics Committee 2013 of the University of Malaya Medical Centre (UMMC). The anticipated benefits are to improve the efficacy of the spermatozoa preparation techniques that includes the procedures of spermatozoa washing, preparation and culture techniques as well as short-term retention of human spermatozoa viability *in vitro*. The improvements is expected to maintain good spermatozoa quality, motility, total count, viability rate, DNA integrity

level and plasma membrane integrity *in vitro* over extended periods of time, post-preparation and after thawing. This would hopefully help in better selection of viable and intact human spermatozoa for assisted reproductive treatments, which will hopefully, increase the fertilization and improve pregnancy rates.

The main objective is to ensure efficient short-term conservation of the spermatozoa viability *in vitro* for up to 24hrs. Intact and viable spermatozoa long hours post-preparation can be used for late insemination especially in intrauterine insemination (IUI). It is hypothesized the stagnant and poor pregnancy rate of about 13% in IUI may be due to inopportune timing of insemination. Most spermatozoa are readily immobilised soon after they are placed in the female reproductive tract (described in greater detail in chapter 5) which suggest that very few motile spermatozoa may be present when ovulation occurs in the IUI patient. One aim of this study is to develop techniques of spermatozoa preparation that would allow retention and viability of the washed spermatozoa for many hours so that insemination can be timed to coincide with ovulation to improve pregnancy rates. This could also be useful in cases when rescue ICSI has to be done a few hours after insemination or the following day when IVF insemination failed to achieve fertilization. In addition, this could be helpful during the *in vitro* maturation of immature metaphase I oocytes, where insemination also need to be done a day or longer after the ovum pick up (OPU). Additionally, it is felt that 24hrs storage of spermatozoa at ambient temperature (i.e. 22^{o}C) would allow for the transportation of the washed samples to other local centres or diagnostic laboratories to standardize laboratory assessments of the semen quality or to conduct additional investigations.

The use of protein-free media in spermatozoa preparation and short term conservation is considered safer as the potential of disease transmission is eliminated compared to commercial protein-containing media.

1.4 OBJECTIVES OF THE STUDY

This is a prospective study using synthetic spermatozoa handling media, aimed to improve methodology for optimal human spermatozoa preparation, as well as to retain the viability of prepared spermatozoa for extended periods of time without cryopreservation.

The specific objectives of this study are:

1. To explore the differences in the efficacy of human spermatozoa handling and washing media in the absence or presence of donor serum albumin on the activity and viability of the harvested spermatozoa.
2. To optimize survival and viability of human spermatozoa over extended periods by modulating the effects of environmental factors in particular incubation temperature and medium.
3. To identify the effects of room temperature and body temperature on human spermatozoa vitality, plasma membrane integrity and DNA fragmentation level.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

This chapter discusses previous studies and investigations that have been made over the last 20 plus years with respect to spermatozoa preparation in ART treatment. It also aims to show how these techniques have dramatically advanced the treatment of both male and female factor infertility, particularly in the development of novel spermatozoa preparation, washing and culturing techniques.

2.2 SPERMATOZOA PREPARATION

The successes of ART treatment would not be possible without the concurrent advances in the laboratory identification, manipulation, and preparation of spermatozoa. Spermatozoa need to be separated from the seminal plasma for a variety of purposes, such as, for diagnostic tests or the therapeutic recovery for ART insemination. However, when the tests of spermatozoa function are to be performed, it is critical that the spermatozoa are separated from the seminal plasma within about an hour after ejaculation (WHO, 2010). This will limit any damage caused by the products of the other cells, dead spermatozoa and other components presented in the semen (Agarwal *et al.,* 1994).

Most of the ART treatments require a selection of the ejaculated spermatozoa before the performance of the artificial insemination. Aitken and Clarkson (1988) have shown that some components of the seminal fluid may become an obstacle when *in vitro* fertilization is performed. Bjorndahl *et al.* (2005), reported that dead spermatozoa, leukocytes and other components (e.g. debris and bacteria) in the semen sample produce oxygen radicals that may alter the quality of spermatozoa. From this point of view, numerous studies and investigations (Araji *et al.*, 2013; Agarwal *et al.,* 1994; Bujan *et al.,* 2007; Fiore *et al.,* 2005; Mortimer, 1990, 1991&1994; Nicopoullos *et al.,* 2010a, b;

Sauer, 2005; Schneider *et al.,* 2014) have developed various washing and preparation techniques that have demonstrated increased efficiency in recovering spermatozoa with improved motility and higher total count. However, Zini *et al.* (1999; 2000) stated that these techniques have some adverse effects on the spermatozoa apoptosis status, mitochondrial function, viability rate, DNA integrity level, spermatozoa surface receptors in addition to alterations on gene expression.

Similarly, Aitken *et al.* (1998) have reported that spermatozoa preparation protocols may affect some features of the spermatozoa and disturb its DNA integrity, as the spermatozoa will be exposed to some environmental insults when they are placed in different washing media and reagents. They have also suggested that these factors may occur as a consequence of environmental changes in the temperature, pressure, light intensity, acidity and the reactive oxygen species (ROS) level, which may subsequently alter the fertility potential of the harvested spermatozoa (Aitken *et al.,* 1998). The work of MacLeod (1943) was the first to show high levels of ROS might disrupt normal spermatozoa function and might also elevate the level of DNA damage. MacLeod also found that human spermatozoa rapidly lose their motility when incubated under high oxygen tensions. Shekarriz *et al.* (1995) found similar results that support the findings Macleod (1943). They have also indicated that ROS production by the spermatozoa increases under certain circumstances, such as from the washing technique and centrifugation. Moreover, Aitken *et al.* (1995) claimed that the presence of leukocytes and dead cells in the *in vitro* incubation media could also be a factor that enhances the ROS production. Furthermore, Loft *et al.* (2003) and Spano *et al.* (2000) have reported that high DNA damage lowers ART outcomes. In addition to that, Evenson *et al.* (2008) have indicated that spermatozoa DNA damage is also highly associated with lower natural pregnancy rates.

The techniques for the selection of the most viable spermatozoa are very important for any clinical practice. Canale *et al*. (1994) reported that the choice of the best technique for semen preparation strictly depends on the quality of the semen sample. For example, when dealing with a normal sample with high spermatozoa counts, motility and morphology, the choice would be the swim-up method. The direct swim-up method was first described more than 30 years ago by Mahadevan and Baker (1984) which was based on the "sperm-rise" technique described by Drevius (1971). Henkel *et al.* (2003) have also reported the efficiency of the swim-up method in recovering motile and viable spermatozoa. It has been known that the spermatozoa swim in all directions in the culture medium including the upper portion in the test tube. This attribute was the basis of the swim-up technique devised by Drevius (1971), the forerunner of the present-day swim-up method. Spermatozoa that swam up the column of medium in the tube are normally clean, free of debris, or dead spermatozoa. Since then, the swim-up technique still the most commonly used technique in IVF laboratories.

Bhattacharya, 1958; Beatty, 1964; Schilling, 1966; Bolton & Braude, 1984; Morrell, 2006 have all described the density gradient centrifugation (DGC) and showed that it is the preferred technique to select greater number of motile spermatozoa in cases of severe oligozoospermia, teratozoospermia or asthenozoospermia. According to the WHO manual (2010), good quality spermatozoa can be separated from dead cells, leukocytes, and the other components of the seminal plasma through the DGC technique. Cells with different density and motility are selected through two different gradients of silica colloidal solutions, either 45 and 90% or 40 and 80%. Spermatozoa with high motility and good morphology are collected at the bottom of the tube. The efficiency of the harvested spermatozoa is expressed as the concentration of the viable spermatozoa with normal motility.

2.3 HUMAN SPERMATOZOA CULTURE

Levran *et al.* (2001) and Van Casteren *et al.* (2008) provided evidence that sometimes during the ART procedure, at the time of OPU procedure of the female partner, the male partner may not be available, or if available, fails to produce semen sample under stressful conditions. In any of these cases, the man can produce the sample one day or many hours before the OPU procedure. Therefore, the spermatozoa will have to be held in culture until the time of insemination that can be many hours post-preparation.

Liu & Zheng (2010) and Nagy *et al.* (2006) suggested that at times when rescue ICSI is needed, insemination would have to be carried out the second day after the sample production and preparation. Rorie (1999) and Van de Velde *et al.* (1998) have also shown that when the retrieved oocytes are mostly immature, the insemination will also have to be performed 24hrs after the OPU procedures. Due to these reasons there is a need to develop spermatozoa holding techniques that could preserve its viability for long periods of time without undergoing detrimental adverse changes due to aging. Lengthy holding of spermatozoa is also likely to cause detriment to the quality of the harvested spermatozoa due to unfavorable conditions created by products of metabolism. An efficient spermatozoa *in vitro* culturing protocol that maintains spermatozoa integrity and viability over extended periods of time post-preparation need be developed. The retention of human spermatozoa throughout the ART procedures is helpful to maintain spermatozoa fertilizing potential during transfer between two distant centers that could avoid the need for cryopreservation. Angelopoulos *et al.* 1999; Ashworth *et al.* 1994; Balaban *et al.* 1999; Gomez & Atiken, 1996; Liu *et al.* 1997; Menegazzo, *et al.* 2011; Moore & Hartman, 1986 & Moore *et al.* 1992 studied different protocols of human spermatozoa culture *in vitro.* Most of these studies showed a satisfactory degree of retention of the clinically relevant spermatozoa parameters.

Ellington *et al.* (1991), and Moore and Hartman (1986) claimed that "some of the cellular functions of human spermatozoa are preserved better through co-culture". Wetzels *et al.* (1991) observed higher motility of human spermatozoa when co-cultured with vero cells for 24hrs at 37˚C in an environment of 5% CO_2 when compared with the sibling spermatozoa cultured without helper cells. Moore *et al.* (1998) showed that human spermatozoa co-incubated with the epithelial cells could improve the progressive motility, zona binding and, in some instances, improved the fertilizing capacity *in vitro.*

Recently, Menegazzo *et al.* (2011) have observed an efficient long-term *in vitro* co-culture of human spermatozoa with isolated Sertoli cells. They have also shown their ability to preserve normal spermatozoa viability, motility and mitochondrial function. It is well known that the Sertoli cells facilitate the progression of germ cells to spermatozoa by controlling the environment within the seminiferous tubules (Griswold, 1998). However, Menegazzo *et al.* (2011) reported that the Sertoli cells could not induce the acrosome reaction or the hyperactivation in their Sertoli cell co-culture system.

Mahi and Yanagimachi (1973) conducted several experiments on the effect of temperature on spermatozoa; a similar study was also performed by Si (1997). Their studies showed that the incubation temperature had a modulatory effect on spermatozoa capacitation and acrosome reaction in animals. White *et al.* (1990) described how the temperature influences the motility, spontaneous acrosome reaction, and the spermatozoa's ability to penetrate the ZP-free hamster egg in humans. Sanshez and Schill (1991) performed further investigations during which they observed the viability and the acrosome status can be preserved better when the washed spermatozoa is incubated at low temperature (4˚C), compared to the conventional incubation temperature (37˚C) in humans. Their findings noted the conventional incubation temperature did not show any significant difference in the penetration rate and the number of decondensing spermatozoa heads.

Marin-Briggiler *et al.* (2002) suggested that human spermatozoa motility depends on a number of factors, including the temperature at which the spermatozoa are kept until the time of insemination. They reported that "Generally, the temperatures lower than 4°C and higher than 37°C have shown some negative effects on the spermatozoa functions based on their observations on motility, penetration into cervical mucus and hamster egg penetration". In another work, Marin-Briggiler *et al.* (2002) showed a significant retention of the capacitation rate of the human spermatozoa after 24hrs of incubation at room temperature (20°C) and their findings of an array of tyrosine phosphorylated proteins normally found in uncapacitated spermatozoa proved the point that room temperature retained the capability of the spermatozoa longer. However, this effect is easily overcome and hyperactivation occurred when the spermatozoa are exposed to 37°C indicating the role of temperature in modulating spermatozoa potential to penetrate and fertilize the oocyte. Esfandiari *et al.* (2002) reported that the exact mechanisms that caused decreased spermatozoa motility and quality at extreme temperatures remains to be elucidated.

These observations led to further investigations that explored the influence of the holding temperature especially room temperature (RT) during the spermatozoa incubation and short-term holding *in vitro*. Thijssen *et al*. (2014) studied the effects of long-term *in vitro* incubation of human spermatozoa at room temperature (RT) versus 35°C, which is to them is the temperature of the testis. They found that the progressive motility and the morphological integrity were significantly higher after 24hrs incubation at RT. Additionally, they reported that the proportions of acrosome reacted apoptotic and dead spermatozoa were significantly lower in the samples incubated at RT compared with 35°C (Thijssen *et al.,* 2014). These findings are similar to an earlier study by Cohen *et al.* (1985), who observed that "room-temperature-stored human spermatozoa were still able

to fertilize human oocytes 5 days after preparation, whereas, the refrigerated sibling spermatozoa showed decline in motility rapidly after 24hrs of storage".

In addition to the previous attempts to maintain the motility and viability of human spermatozoa *in vitro* post-wash, Matson and Tardif (2014) investigated further the effects of the macromolecules to support short-term spermatozoa culture. However, they documented that these macromolecules are well proven to be unsatisfactory for clinical use, due to their physical properties.

Many studies and investigations have been performed to retain the quality and motility of human spermatozoa and to maintain their fertilizing potential long hours after washing. However, there is yet an efficient technique of less or no adverse effects for short-term maintenance of spermatozoa activity have been devised which is urgently needed as it would help to improve the fertilization rate of human spermatozoa and the success rate of the ART treatment.

2.4 HUMAN SPERMATOZOA CULTURE AND HANDLING MEDIA

At the present moment it appears that all commercial cell and embryo culture media are comes supplemented with donor serum proteins (DSPs) which is an essential component for spermatozoa capacitation (Parrish *et al.,* 1985; Dinkins and Brackett, 2000) and survival (Critser *et al.*, 1984). Leung *et al*. (1984) stated that besides DSPs, a variety of other contaminant compounds and molecules are also presented in the culture media supplemented with DSPs, because the contents of the media varies with each batch creating batch variation and inconsistency in the content of the culture media due to the differences in the donor populations. Until the early 1990s, the most commonly used protein source in ART programs was the human serum, obtained from the patient herself. However, Alberda *et al*. (1989) noted that pooled donor serum has a high risk of transmitting infections and hepatitis infections in ART has been reported (Van Os *et al*.,

1991). There has even been an incidence of potential transmission of Variant Creutzfeldt-Jakob disease (vCJD) (Kemmann, 1998). The realty of the situation is that biological supplements such as serum proteins cannot be sterilized with certainty (Truyen *et al.*, 1995). In addition, Leveille *et al.* (1992) and Dokras *et al.* (1993) have shown that some patients were found to have embryo toxic factors in their serum. Therefore, more attention has been given to reduce the risk of infection or toxicity from blood-derived products within ART programs. This is also to avoid a variety of other unknown contaminant components of that might interfere the ART treatment procedures.

Numerous investigations have since then been carried out to seek alternative sources of proteins that could replace the human serum albumin (HSA) in the ART procedures as well as during the artificial breeding of the domestic animals for food production and exotic species. One of the earliest report of embryo culture in a defined medium without DSPs was that of Cholewa and Whitten in 1965. Culture media devoid of added proteins have also been developed for mouse, rabbit and primates have been reported (Spindle, 1995; Li *et al.*, 1996; Schramm & Bavister, 1996). Earlier Biggers *et al.* (1997) investigated the effect of replacing serum albumin with polyvinyl alcohol (PVA) and/or amino acids on mouse zygote development. They observed that PVA could not be substituted completely for serum albumin in the mouse embryo culture medium. Parinaud *et al.* (1998) utilized papain, a plant enzyme to strip cumulus cells off human oocytes in an attempt to avoid the bovine-derived enzyme hyaluronidase that carries risk of disease transmission. Caro and Trounson (1986) and Parinaud *at al.*, 1999 also developed protein-free embryo culture media for human ART, but these workers could not overcome the need for proteins in their spermatozoa preparation medium that is required for spermatozoa capacitation. Parinaud *et al.* (1999) noted reduced spermatozoa motility in their spermatozoa preparation medium which they suggested might be due to some unknown stimulatory molecules in the bovine serum albumin which they apparently

had used in their spermatozoa handling medium. In short while their embryo culture medium was protein-free but their spermatozoa preparation contained DSPs. Their media system was therefore not completely synthetic. Other workers have replaced serum protein with a single antioxidant and chelator such as EDTA (Mehta & Kiessling, 1990; Serta *et al*., 1997) in embryo culture medium that does not impair fertilization and cleavage of viable embryos in the mouse and human, but it appears their embryos stuck to the sides of dishes and catheters.

In another attempt the recombinant human albumin (rHA) was used as an alternative to the HSA. Gardner and Lane (1999) carried out the first trial on mouse embryos, and found that fertilized oocytes cultured in media supplemented with a concentration of 1.25 mg/ml rHA produced equivalent rates of blastocyst development, and equivalent cell number in the inner cell mass and trophectoderm as the embryos cultured with 5mg/ml HSA. Bungum *et al.* (2002) eventually performed the rHA supplementation on humans. The results showed that human embryos cultured in a medium containing either the rHA or the conventional HSA produced comparable rates of fertilization, cleavage, blastocyst formation and implantation, as well as pregnancy loss. Bavister *et al.* (2003) support these findings and have also studied the efficiency of the rHA in the human spermatozoa culturing and handling media. It was shown that the rHA supplementation has an equivalent efficiency to the commercial HSA supplemented human spermatozoa culturing media. In addition, Bavister *et al.* provided evidence of the ability of the rHA to support spermatozoa capacitation and fertilization rates. However, the rHA is a high cost product, which is presently prohibitive; besides the industry appears to be of the opinion the consistency of the rHSA is difficult to maintain.

Subsequently, a completely synthetic media series free of donor albumin media have been described for the first time (Ali, 1997, 2001 & 2004; Ali *et al.*, 2000) that was efficacious for human embryos with excellent outcome. Ali developed the protein-free

human culture media series (PFM), which is able to support both fertilization and embryo development. As detailed in its patent (Ali, 2007), the PFM contained completely defined supplements that has been observed to generate viable human embryos. The PFM media could support approximately 80% spermatozoa survival over 24hrs at 37°C (Ali *et al.*, 2000).

Furthermore, Kim *et al.* (2007) reported that the macromolecules derived from non-animal sources such as the PVP, PVA or FBS can all be used as an alternative to BSA for supplementation of the basal medium. Matson and Tardifc (2014) carried on several studies to investigate the performance of the macromolecules as an alternative to the donor serum albumin. They have shown efficiency of macromolecules in supporting short-term spermatozoa culture, also did not significantly affect the rate of spontaneous acrosome reaction when compared to the bovine serum albumin supplemented media but they noted that some macromolecules may well prove unsatisfactory for clinical use (Matson & Tardif, 2014).

Other more recent studies in support of the need for synthetic media protein-free have demonstrated the presence of numerous protein contaminants in HSA used in embryo culture media that could be potentially harmful (Dyrlund *et al.*, 2014). The amount of these contaminant proteins which numbered about 110 different proteins varies with each batch of culture medium contributing to batch variation and inconsistency in the quality of the embryo culture media manufactured. Of interest is that Drylund *et al.* (2014) noted that these “undeclared” contaminant proteins present in unconditioned media could potentially influence embryonic development, gestation age, birthweight and perhaps have subsequent effects on health of the offspring. A potentially more insidious effect of using HSA in human embryo culture media for ART treatment is the potential risk of crossover of the embryonic genome by the contaminating micro DNA and micro RNA strands found in HSA. This is because the embryo consumes culture medium by

pinocytosis (Fleming & Pickering, 1985). Consequently it may ingest whole contaminant donor micro DNA and Micro RNA strands from the embryo culture medium which could lead to a myriad of genetic anomalies. For instance imprinting disorders have been noted in cultured embryos (Khosla *et al.,* 2001; Rivera *et al.,* 2008; Market-Velker *et al.,* 2010; Catchpoole *et al.,* 2000; Halliday *et al.,* 2004; Chang *et al.,* 2005; Dumoulin *et al.,* 2010; Nelissen *et al.,* 2012). These findings and suggestions indicated for the need to develop synthetic culture media not only in the ART but in all areas of medicine where culture media containing DSPs are in use at the present times.

CHAPTER 3: MATERIALS AND METHODS

3.1 INTRODUCTION

The objectives of study was to improve spermatozoa washing and preparation techniques. In addition, the study aims to improve the retention of viability of human spermatozoa *in vitro* over extended periods of time. This study was conducted from February 2014 through June 2015 with approval of the Ethics Committee of the University of Malaya Medical Centre (Ethics/IRB Ref. 1073.52).

All laboratory work was performed at the Clinical Research Laboratory, Department of Obstetrics and Gynaecology at the University of Malaya Medical Centre (UMMC). This chapter will detail the commercial sources of the specialized spermatozoa culture and washing media. In addition, this chapter will discuss in detail the procedures employed for human semen collection and analysis, spermatozoa evaluation, preparation of culture and handling media and spermatozoa washing, preparation and culture techniques. The experimental design of each experiment is also described.

3.2 MATERIALS

3.2.1 Human Semen Samples

Human semen samples were obtained from 249 men undergoing semen assessment at the University of Malaya Fertility Centre (UMFC); the Polyclinic at the University of Malaya Medical Centre (UMMC), the Infertility Unit at the Hospital Kuala Lumpur (HKL) and from the human reproductive unit at the Lembaga Penduduk dan Pembangunan Keluarga Negara (LPPKN), which are located in Kuala Lumpur, Malaysia.

3.2.2 Reagents and Culture Media

The commercial protein-containing media selected for this study are: the Multipurpose Handling Medium™ (Catalog ID: 90163), Continuous Single Culture Medium™ (Catalog ID: 90164), and Human Serum Albumin Solution (HSA) (Catalog ID: 9988), which have been ordered from IRVINE SCIENTIFIC (USA). The commercial synthetic protein-free media used in the study are: the Protein-Free IUI Medium with HEPES and Gentamycin (Catalog ID: GP40050), Protein-Free Human Embryo Culture Medium with Gentamycin (Catalog ID: EM10050), Protein-Free GradiART Lower Layer (Catalog ID: GP2105) and the Protein-Free GradiART Upper Layer (Catalog ID: GP31050) from CELLCURA ASA (Norway). The Minimum Essential Medium (MEM) with HEPES and the MEM without HEPES are obtained from BIOWEST SAS (France).

3.3 METHODS

3.3.1 Human Semen Sample Collection and Analysis

After 2-3 days of sexual abstinence semen samples were produced by masturbation and analysed by standard methods (WHO 2010). Human semen samples were collected in a private room near to the laboratory, in order to limit the exposure of the semen to fluctuations in temperature and other environmental factors as well as to control the interval between semen sample collection and analysis. A written informed consent was obtained from each patient that agreed to participate in the study. Each participant was given a clear written and spoken instruction concerning the method of semen sample collection.

Semen analysis was performed according to the guidelines of the WHO 2010 Format. The semen analysis examination includes: semen appearance: the condition of semen in the ejaculate (e.g: colour, volume: the quantity of the semen present in one ejaculation, liquefaction time: the time it takes for the semen to liquefy, spermatozoa

concentration: the number of spermatozoon present per millilitre (ml) of the semen in one ejaculation, morphological features: the percentage of spermatozoa of a normal characteristics exist in the semen sample in one ejaculate, vitality: percentage of the live spermatozoa in the semen sample of one ejaculate, spermatozoa motility: the percentage of spermatozoa that can move forward normally, and pH: the measurement of the acidity (low pH) or alkalinity (high pH) of the semen sample).

Other information recorded in the semen analysis form of each patient include: the patient's full name, date of birth, the days of abstinence, the date and time of semen collection as well as the interval between semen sample collection and semen analysis.

3.3.2 Evaluation of Human Spermatozoa Vitality and Viability

3.3.2.1 Evaluation of Spermatozoa Motility

A modified Neubauer counting chamber (Hawksley, Lancing, England) was used to assess the total spermatozoa motility as stated in the WHO Semen Analysis Manual 2010. Observations were made using a light microscope at 200x magnification. A total of 200 spermatozoa counts were taken. Readings were expressed as the percentage of motile spermatozoa in the sample, (%) = (number of motile spermatozoa x 100)/ (n=200).

Due to the propensity of the heads of spermatozoa prepared using the PFM to stick to the surfaces of the counting chamber or dishes a holding time of 5-7mins at 37^{o}C is required after charging the Neubauer chamber. This would enable the spermatozoa detach themselves before the readings were taken as recommended by the inventor of the PFM (Ali, 1997). More recently Peirce *et al.* have also noted this phenomenon peculiar to PFM media but could not relate it to static electricity as originally suggested by the inventor of the PFM. They noted a 6mins wait allowed spermatozoa detach themselves from the chamber and permitted free mobility. They therefore have recommended a 6mins wait before readings are taken (Peirce *et al.,* 2015). To standardize the spermatozoa motility

evaluation method all prepared spermatozoa irrespective of the washing or culture media were held for 5-7mins prior to motility evaluation.

3.3.2.2 Evaluation of Spermatozoa Vitality

Spermatozoa VitalStain™ (SVS, Nidacon International AB, Mölndal, Sweden) was used to differentiate the live spermatozoa from the dead. The VitalStain™ solution contains both eosin and nigrosine stains. The test was performed according to the protocol provided by the manufacturer.

Equal amounts of the VitalStain™ solution and spermatozoa sample (50µl SVS+50µl spermatozoa sample) were mixed well in an Eppendorf micro tube. The mixture was kept for 30sec at room temperature prior to observation. A total of 200 spermatozoa were counted per observation. If the spermatozoa absorbed the stain and coloured red, they are considered as dead. However, if the spermatozoa remained unstained and shone, they were recorded as live spermatozoa. Human spermatozoa vitality is the percentage of the live spermatozoa in the semen sample.

3.3.2.3 Evaluation of Human Spermatozoa Plasma Membrane Integrity

The hypo-osmotic swelling (HOS) test was performed to get an assumption of human spermatozoa plasma membrane integrity. This test was performed as described by its originator (Jeyendran *et al*., 1984). The test was performed by mixing 0.1ml of the spermatozoa sample with 1ml of the hypo-osmotic solution. The mixture was incubated at 37°C for at least 30mins. A modified Neubauer counting chamber (Hawksley, Lancing, England) was charged with the treated spermatozoa and allowed to stand for at least 5mins prior to observation was made under the phase-contrast microscopy at 400x magnification. A total of 200 spermatozoa were observed and evaluated per each observation. The percentage of spermatozoa with swollen tail were calculated.

$$\text{Membrane Integrity Level (\%)} = \frac{\text{Number of Spermatozoa with Swollen Tail x 100}}{\text{Total Number of Spermatozoa Counted}}$$

The presence of spermatozoa with swollen tail is a suggestion of the functionality and integrity of their plasma membrane and thus it is considered as an assumption of the spermatozoa viability (Jeyendran *et al*., 1984). The proportion of spermatozoa with swollen tail obtained in the treated spermatozoa is an indication of the capability of the treatment to retain the viability and functionality of the human spermatozoa plasma membrane compared to other treatment protocols.

3.3.2.4 Evaluation of Human Spermatozoa DNA Integrity

Spermatozoa DNA fragmentation (SDF) level was measured using the Halosperm® G2 kit (HALOTECH DNA, Madrid, Spain). The assay was performed according to the instructions of the manufacturer.

The post-wash human spermatozoa samples were diluted in a culture medium to a concentration of $20x10^6$ per 1ml. The agarose gel provided in the kit was dissolved in the water bath at 90-100^{o}C. It is then maintained at 37^{o}C in a water bath for 5mins until the temperature equilibrated. Spermatozoa solution (50µl) was added to 100µl of the melted agarose and mixed well; the temperature was maintained at 37^{o}C. Spermatozoa-agarose mixture (8µl) was placed in the centre of the well on the glass slide and covered with a coverslip (air bubble formation was avoided). The slide was placed on a cold surface and kept in the refrigerator at 4^{o}C for 5mins to allow the agarose solidify. The slide was then kept at room temperature along the rest of the process. The solidified agarose-spermatozoa mixture was treated with the denaturant agent then the lysis solution for 7mins and 20mins respectively. The slide was washed for 5mins with distilled water

then immerged into 70% ethanol for 2mins to dehydrate the spermatozoa. This was followed by 100% ethanol for 2mins. The slide was finally air dried prior to the staining as described by the manufacturer.

$$\text{Spermatozoa DNA Integrity Level (\%)} = \frac{\text{Number of Spermatozoa with Halo x 100}}{\text{Total Number of Spermatozoa Counted}}$$

The presence of a halo of chromatin decondensation around the spermatozoon head is indicative of an intact DNA, however, the absence of the halo is sign of a damaged DNA. The visualization was performed under bright field microscopy at 400x magnification. A total of 200 spermatozoa were counted for each observation. A negative control (spermatozoa without halo) was performed alongside each sample tested.

3.3.3 Human Spermatozoa Washing and Preparation Technique

Spermatozoa was washed by the standard density gradient centrifugation (DGC) technique. The DGC allow separation of good quality spermatozoa from dead spermatozoa, leukocytes and other components present in the seminal fluid. The centrifugation steps separate cells based on their buoyant density. Live human motile spermatozoa and good morphology can be obtained at the bottom of the centrifugation tube.

The DGC tube is prepared by layering 2ml of 40% gradient solution over a 2ml layer of 80% gradient solution (Protein-Free GradiART, Cellcura ASA, Norway) in a 15ml conical tube. Liquefied semen sample was layered over the upper layer of the gradient solution and centrifuged for 20mins at 1500rpm at room temperature. After the centrifugation, most of the supernatant was gently removed and the pellet is placed into a new and clean 15ml conical tube for the second washing step. The pellet obtained at the

bottom of the centrifugation tube was re-suspended in 3ml of HEPES buffered spermatozoa handling media (Cellcura ASA, Norway) and re-centrifuged at 1500rpm for 5mins to wash away the remaining gradient solutions and traces of the seminal plasma from the harvested spermatozoa. At the end of the final washing and centrifugation, the supernatant was placed in a new tube of spermatozoa culture media, to be cultured and apportioned for various treatments.

3.3.4 Preparation of the Minimum Essential Medium (MEM)

The minimum essential medium was obtained from Sigma Chemicals (USA) with minor modifications. The modifications made were the incorporation of additional components (per 100ml of final solution) as listed below:

- Sodium Pyruvate (0.002975g).
- Sodium Lactate (0.19ml).
- Human Serum Albumin (HSA) (5ml).

The MEM handling medium contained in addition to the above, HEPES (0.594g in 100ml). The modified MEM culture and handling media were adjusted to about 285mOsm, filtered sterile using a 0.22micron filter kit (Corning® Incorporated, Tewksbury, USA), apportioned into 50ml sterile flasks, shielded from light and stored in a refrigerator at 4˚C. Fresh media were prepared every 16 weeks.

3.3.5 Preparation of the Hypo-Osmotic Solution

This medium was prepared based on the method developed by Jeyendran *et al*. (1984). The hypo-osmotic solution was prepared by adding Sodium Citrate.$2H_2O$ (7.35g) and Fructose (13.51g) in distilled H_2O (1000ml). The solution was apportioned into 50ml flasks, labelled and dated. The solution was kept at 4^oC in the refrigerator or frozen for long durations at -20^oC. The hypo-osmotic medium was warmed to room temperature

prior to usage. Subsequently, 0.1ml of the spermatozoa sample was added to 1ml of the hypo-osmotic solution and incubated for 30mins prior to observation. A total of 200 spermatozoa were observed at each reading taken.

3.4 EXPERIMENTAL DESIGN

3.4.1 (EXPERIMENTS 1a-b) EFFECT OF HANDLING AND CULTURE MEDIA WITH AND WITHOUT SERUM PROTEINS ON HUMAN SPERMATOZOA ACTIVITY

Normozoospermic semen samples were washed and sibling spermatozoa cultured for 4-7hrs and 24hrs using: (i) Synthetic Protein-Free Media (PFM, Cellcura ASA, Norway), (ii) Minimum Essential Medium + HSA (MEM, Biowest SAS, France) and (iii) Commercial Protein-Containing Medium (CPC; Irvine Scientific, USA). Sibling spermatozoa were equally apportioned and subsequently cultured *in vitro* in 1.0-0.5ml media in culture tubes with loosen cap and placed inside the inclubator with a gas mixture (6% CO_2 in air) for 24 hrs. Human spermatozoa were evaluated for their motility, vitality, plasma membrane integrity and the spermatozoa DNA fragmentation (SDF) levels at 0hr post-wash (Expt 1a) and after 4-7hrs and 24hrs post-wash (Expt 1b).

3.4.2 (EXPERIMENTS 2a-b) EFFECT OF DIFFERENT INCUBATION TEMPERATURES AND CULTURE MEDIA ON HUMAN SPERMATOZOA MOTILITY *IN VITRO*

In Experiment 2a, washed human spermatozoa were apportioned equally into three main parts. Individual portions were held at 4˚C, room temperature (22˚C) or 37˚C. Spermatozoa were cultured using different culture media (PFM, MEM or CPC) for up to 24hrs *in vitro*. The total motility of human spermatozoa were evaluated at 4-7hrs and after 24hrs post-wash. A total of 25 human semen samples were used in this experiment. In

Experiment 2b, an additional holding temperature of 15^{o}C was included while all other conditions remained identical to Expt 2a. Prior to incubation at different temperatures, samples of washed spermatozoa were gassed with a gas mixture (6% CO_2 in air) for 5mins and sealed airtight to maintain the pH of the culture medium.

3.4.3 (EXPERIMENT 3): VITALITY AND VIABILITY OF HUMAN SPERMATOZOA HELD AT ROOM TEMPERATURE USING VARIOUS CULTURE MEDIA

In Experiment 3, human spermatozoa vitality, membrane integrity and the DNA fragmentation (SDF) levels were evaluated at 22^{o}C (the optimal temperature obtained in Expt 2b) and compared to that at 37^{o}C (Control) in PFM, MEM and CPC. The evaluation was performed at 4-7hrs and after 24hrs post-wash as described in section 3.3.2. A total of 21 human semen samples were used in this experiment.

3.5 STATISTICAL ANALYSIS

Statistical analyses was performed using the IBM SPSS Statistical Software Version 22 (IMB, International Business Machines Corp, New York 10504). The results are presented as means of proportions. The data obtained were checked for homogeneity and the distribution of the data through the same statistical software. The difference between various factors were evaluated using ANOVA. The differences were considered significant when $p<0.05$. The differences between groups of variables were calculated through the post-hoc test (Two-Way ANOVA/Duncan). Pairwise, comparisons between the two means of parameters were determined using the paired student's t-test using the Statistix^{TM} statistical software (Statistix version 10, USA)

CHAPTER 4: RESULTS

4.1 INTRODUCTION

This chapter presents the findings obtained from the experiments performed in this study. The results are displayed in the form of tables and graphs. The data are presented as means of percentages (%) or as means of percentages ± standard error for all parameters. The differences between treatments are considered statistically significant when $p<0.05$.

4.2 (EXPERIMENTS 1a-b) EFFECT OF HANDLING AND CULTURE MEDIA WITH AND WITHOUT SERUM PROTEINS ON HUMAN SPERMATOZOA ACTIVITY

The effect of synthetic protein-free medium (PFM), minimum essential medium + serum proteins (MEM) and the commercial protein-containing medium (CPC) on post-wash spermatozoa activity and viability immediately after preparation (Expt 1a) is given in Table 4.1. There were no significant differences between PFM, MEM and CPC washing media with regard to spermatozoa motility ($p=0.206$); vitality ($p=0.315$); plasma membrane integrity ($p=0.401$) or spermatozoa DNA integrity level ($p=0.587$).

Likewise, after 4-7hrs of *in vitro* culture (Expt 1b), no significant difference was noted with regard to the effect of PFM, MEM and CPC culture media on human spermatozoa motility ($p=0.891$), vitality ($p=0.848$), plasma membrane integrity ($p=0.134$) and DNA integrity level ($p=0.182$). The same findings were seen after 24hrs *in vitro* culture, when the same parameters were evaluated; motility ($p=0.490$), vitality ($p=0.662$), plasma membrane integrity ($p=0.557$) and the DNA integrity level ($p=0.661$) (Table 4.2).

Table 4.1: Effects of various spermatozoa washing media on human spermatozoa activity and viability at 0hr post-wash

Wash Media	Motility	Vitality	Membrane Integrity	DNA Integrity
PFM N=25	(57* ± 4.39)	(76* ± 5.84)	(77* ± 3.43)	(98* ± 1.84)
MEM N=25	(51* ± 3.49)	(68* ± 5.29)	(69* ± 4.49)	(98* ± 1.55)
CPC N=25	(61* ± 5.31)	(69* ± 5.83)	(74* ± 4.65)	(99* ± 0.91)

(PFM) protein-free medium, (MEM) minimum essential medium, (CPC) commercial protein-containing washing medium.
Results expressed as means of percentage ± standard error. Date obtained from 25 men.
(*****) $p>0.1$; when the means of each parameter were compared to that of sibling spermatozoa washed using different wash media.

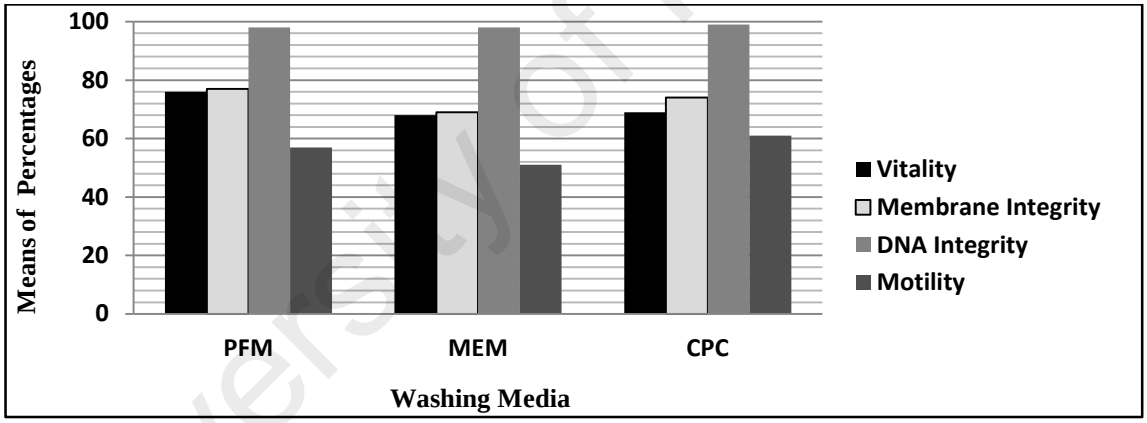

Figure 4.1: Effect of protein-free medium (PFM), minimum essential medium (MEM) and the commercial protein-containing washing medium (CPC) on human spermatozoa vitality, plasma membrane integrity, DNA integrity level and motility at 0hr post-wash; (N=25). No significant differences ($p>0.1$) were noted between the means of all parameters when compared to those of sibling spermatozoa washed using different spermatozoa handling media.

Table 4.2: Effects of various spermatozoa culture media on human spermatozoa activity and viability at 4-7hrs and 24hrs post-wash *in vitro*

Culture Media	4-7hrs				24hrs			
	Motility	Vitality	Membrane Integrity	DNA Integrity	Motility	Vitality	Membrane Integrity	DNA Integrity
PFM N=25	(56* ± 3.78)	(71* ± 4.43)	(58* ± 4.68)	(92* ± 1.20)	(31* ± 5.82)	(54* ± 5.59)	(43* ± 4.95)	(86* ± 3.61)
MEM N=25	(48*± 3.72)	(67* ± 4.36)	(58* ± 3.51)	(90* ± 2.00)	(26* ± 4.49)	(54* ± 5.30)	(44* ± 3.46)	(86* ± 2.89)
CPC N=25	(58* ± 3.57)	(71* ± 4.42)	(59* ± 2.71)	(96* ± 0.88)	(32* ± 5.45)	(54* ± 4.67)	(39* ± 4.09)	(89* ± 4.04)

(PFM) protein-free medium, (MEM) minimum essential medium, (CPC) commercial protein-containing culture medium.
Results expressed as means of percentage ± standard error. Data obtained from 25 men.
(*****) $p>0.1$; when the means of each parameter at 4-7hrs and 24hrs post-wash were compared with those of sibling spermatozoa cultured using different culture media.

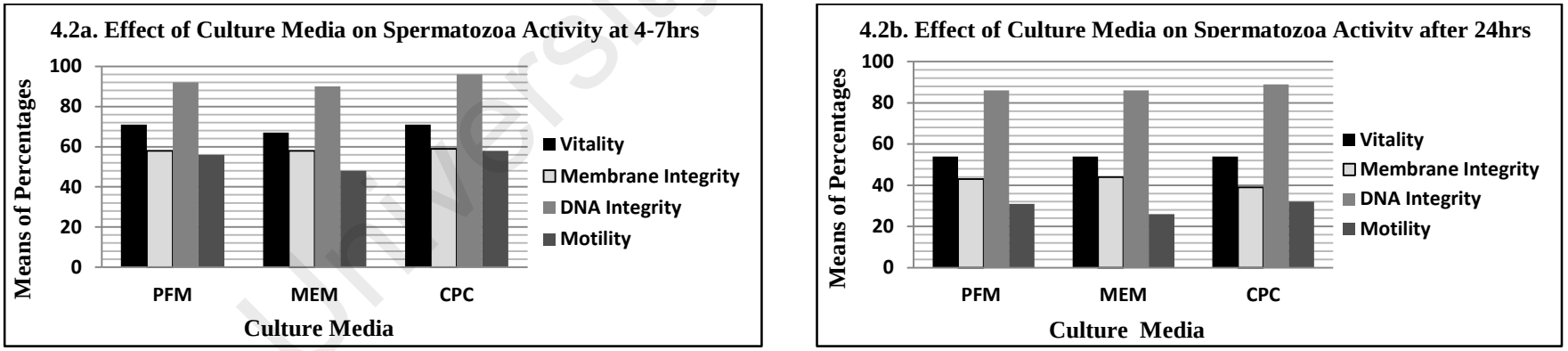

Figure 4.2: Effect of PFM, MEM and CPC culture media on human spermatozoa activities: (4.2a) at 4-7hrs and (4.2b) after 24hrs post-wash; (N=25). No significant difference ($p>0.1$) in all parameters when spermatozoa were cultured using different culture media up to 24hrs culture.

4.3 (EXPERIMENTS 2a-b): EFFECT OF DIFFERENT INCUBATION TEMPERATURES AND CULTURE MEDIA ON HUMAN SPERMATOZOA MOTILITY *IN VITRO*

In this experiment washed human spermatozoa were incubated at different holding temperatures (4^{o}C, 15^{o}C, 22^{o}C and 37^{o}C) using PFM, MEM or CPC culture medium. In Experiment 2a, the motility of washed human spermatozoa was evaluated at 4^{o}C, 22^{o}C and 37^{o}C after 24hrs *in vitro* incubation. However, in Experiment 2b another holding temperature, "15^{o}C", was incorporated into the study (Expt 2a).

The highest human spermatozoa motility was obtained at 22^{o}C, which was significantly higher than 4^{o}C (p<0.05), but different (p>0.05) when compared to that at 15^{o}C and 37^{o}C at 4-7hrs post-wash in PFM, MEM and CPC culture medium. Likewise, after 24hrs post-wash, human spermatozoa motility was highest at 22^{o}C and it was significantly higher (p<0.05) than that at 4^{o}C, 15^{o}C and 37^{o}C in all culture media (Tables 4.4).

At 4-7hrs, the motility of washed human spermatozoa at 22^{o}C appeared to be not significantly different (p>0.05) with the initial motility recorded at 0hr post-wash. In contrast, the motility at 4^{o}C, 15^{o}C and 37^{o}C was significantly different (p<0.05) when compared to the initial motility. Moreover, after 24hrs human spermatozoa motility at all holding temperatures was significantly different from the initial motility obtained at 0hr post-wash in all culture media (Table 4.4).

Table 4.3 Effects of various spermatozoa culture media and temperature (4˚C, 22˚C and 37˚C) on human spermatozoa motility at 4-7hrs and 24hrs post-wash *in vitro*

Culture Media	Holding Time (Hour)	37˚C	22ºC	4ºC
PFM N=25	0hr	$54^{(a)}$	$54^{(a)}$	$54^{(a)}$
	4-7hrs	$41^{(a,b)}$*	**47**$^{(b)}$	$39^{(a)}$‡
	24hrs	$23^{(c)}$‡	$45^{(b)}$†	$12^{(a)}$‡
MEM N=25	0hr	$50^{(a)}$	$50^{(a)}$	$50^{(a)}$
	4-7hrs	$43^{(a,b)}$†	**45**$^{(b)}$	$34^{(a)}$‡
	24hrs	$8^{(c)}$‡	$35^{(b)}$†	$5^{(a)}$‡
CPC N=25	0hr	$59^{(a)}$	$59^{(a)}$	$59^{(a)}$
	4-7hrs	$49^{(a,b)}$†	**55**$^{(b)}$	$41^{(a)}$‡
	24hrs	$27^{(c)}$‡	$51^{(b)}$‡	$14^{(a)}$‡

(PFM) protein-free medium, (MEM) minimum essential medium, (CPC) commercial protein-containing culture medium. Results are expressed as means of the motility percentage. Data obtained from 25 men.

Means of different superscripts $^{(a,\ d,\ c)}$ at each holding durations are significantly different (p<0.05).

(*) p<0.05, (†) p<0.01, (‡) p<0.001, and the bold values (p>0.05) when the motility at 4-7hrs and 24hrs was compared individually with the initial motility in all culture media.

The means of motility in each row were not significantly different (p>0.05), when compared to those cultured in different culture media.

Table 4.4: Effect of various spermatozoa culture media and temperature (4˚C, 15˚C, 22˚C and 37˚C) on human spermatozoa motility at 4-7hrs and 24hrs post-wash *in vitro*

Culture Media	Holding Time (Hour)	Holding Temperature			
		37˚C	22ºC	15ºC	4ºC
PFM N=25	0hr	$52^{(a)}$	$52^{(a)}$	$52^{(a)}$	$52^{(a)}$
	4-7hrs	$40^{(a,b)}$*	**47**$^{(b)}$	$41^{(a,b)}$*	$36^{(a)}$‡
	24hrs	$22^{(b)}$‡	$43^{(c)}$*	$33^{(b)}$†	$15^{(a)}$‡
MEM N=25	0hr	$48^{(a)}$	$48^{(a)}$	$48^{(a)}$	$48^{(a)}$
	4-7hrs	$41^{(a,b)}$†	**44**$^{(b)}$	$38^{(a,b)}$†	$33^{(a)}$‡
	24hrs	$18^{(b)}$‡	$37^{(c)}$†	$28^{(b)}$‡	$8^{(a)}$‡
CPC N=25	0hr	$57^{(a)}$	$57^{(a)}$	$57^{(a)}$	$57^{(a)}$
	4-7hrs	$49^{(a,b)}$†	**55**$^{(b)}$	$49^{(a,b)}$†	$40^{(a)}$‡
	24hrs	$27^{(b)}$‡	$49^{(c)}$†	$41^{(b)}$‡	$12^{(a)}$‡

(PFM) protein-free medium, (MEM) minimum essential medium, (CPC) commercial protein-containing culture medium. Results are expressed as means of the motility percentage. Data obtained from 25 men.

The means of different superscripts $^{(a, d, c)}$ at each holding duration are significantly different (p<0.05).

(*) p<0.05, (†) p<0.01, (‡) p<0.001, and the bold values (p>0.05) when the motility at 4-7hrs and 24hrs was compared individually with the initial motility in all culture media.

The means of motility in each row were not significantly different (p>0.05), when compared to those cultured in different culture media.

4.4 (EXPERIMENT 3): VITALITY AND VIABILITY OF HUMAN SPERMATOZOA HELD AT ROOM TEMPERATURE USING VARIOUS CULTURE MEDIA

The viability of human spermatozoa was evaluated at 22ºC (best holding temperature obtained in Expt 2b) compared to the control (37ºC). The vitality were significantly higher (p<0.05) at 22ºC than that at 37ºC in PFM, MEM and CPC at 4-7hrs and also after 24hrs post-wash (Table 4.5). However, a significant difference (p<0.05) was noted when the vitality of both 22˚C and 37˚C were compared individually with the initial vitality obtained at 0hr post-wash in all culture media at 4-7hrs and also after 24hrs

of *in vitro* holding duration. Nevertheless, no significant difference ($p>0.05$) was noted when the vitality at both 22ºC and 37ºC were compared to that of different culture media for 4-7hrs and also after 24hrs (Figure 4.3).

The plasma membrane integrity level of washed human spermatozoa was likewise significantly higher ($p<0.05$) at 22ºC than that at 37ºC in PFM, MEM and CPC culture medium at 4-7hrs and 24hrs post-wash (Table 4.6). At 4-7hrs however, the means of membrane integrity at 37˚C were significantly lower ($p<0.05$) than the original reading (at 0hr post-wash) in all culture media. In contrast, the membrane integrity means at 22˚C were found to be not significantly different ($p>0.05$) when compared to the initial integrity level obtained at 0hr post-wash in PFM and CPC culture media, but the difference was not significant ($p<0.05$) in the MEM culture medium. On the other hand, after 24hrs of holding duration, there was a significant difference ($p<0.05$) in the membrane integrity level at both 22˚C and 37˚C when compared to the initial plasma membrane integrity level in all three culture media (Figure 4.4).

Furthermore, there were no significant differences ($p>0.05$) in the means of human spermatozoa DNA fragmentation (SDF) levels when spermatozoa were held at both room temperature (22˚C) and 37˚C for 4-7hrs and also up to 24hrs post-wash using PFM, MEM and CPC culture medium (Table 4.7). Indeed, when the DNA integrity levels at 22˚C were compared to the original level obtained at 0hr post-wash, no significant differences were noted ($p>0.05$) in PFM, MEM and CPC at 4-7hrs and also after 24hrs post-wash. However, at 37˚C there was a significant reduction in the DNA integrity level of spermatozoa held in MEM culture media following 24hrs *in vitro* incubation, whereas, no significant difference was noted in PFM and CPC culture medium (Figure 4.5).

Table 4.5: Effect of various human spermatozoa culture media and holding temperature (22ºC & 37ºC) on human spermatozoa vitality *in vitro*

Culture Media	Holding Temperature	Holding Duration (Hour)		
		0hr	4-7hrs	24hrs
PFM N=21	37˚C	80	$70^{(s)}$	$54^{(s)}$
	22ºC	80	$76^{(s)}$‡	$67^{(s)}$‡
MEM N=21	37˚C	77	$67^{(s)}$	$54^{(s)}$
	22ºC	77	$74^{(s)}$‡	$62^{(s)}$†
CPC N=21	37˚C	78	$70^{(s)}$	$54^{(s)}$
	22ºC	78	$75^{(s)}$*	$63^{(s)}$*

(PFM) protein-free medium, (MEM) minimum essential medium, (CPC) commercial protein-containing culture medium.
Results are expressed as means of vitality percentages. Data obtained from 21 men.
(*) $p<0.05$, (†) $p<0.01$, and (‡) $p<0.001$; when the vitality at 22ºC was compared individually to that held at 37˚C at each holding duration in all culture media.
(s) $p<0.05$; when the vitality at 4-7hrs and 24hrs post-wash was compared with the initial vitality obtained at 0hr post-wash.
The means of human spermatozoa vitality were not significantly different ($p>0.05$) when compared to those cultured using different culture media.

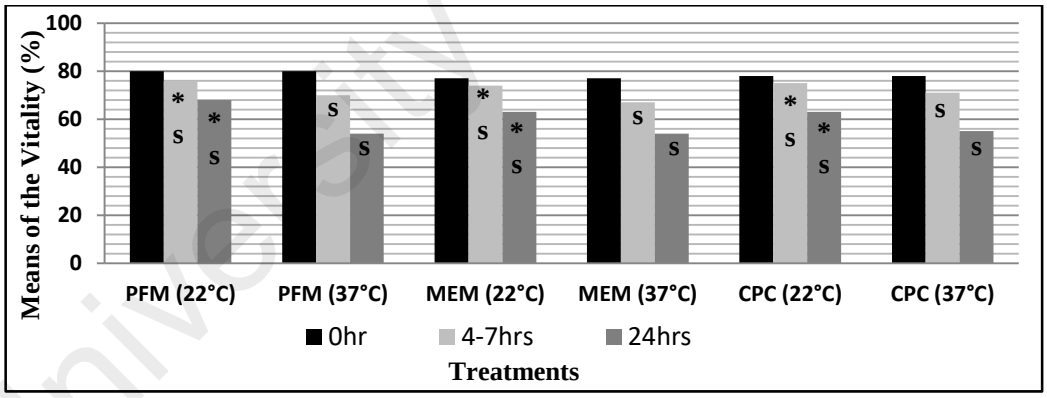

Figure 4.3: Effect of different culture media (PFM, MEM & CPC) and temperature (22˚C & 37˚C) on washed spermatozoa vitality at 4-7hrs and after 24hrs incubation *in vitro*; (N=21).

(*) $p<0.05$; when the vitality at 22˚C compared with the control (37˚C) in all culture media.

(s) $p<0.05$; when the vitality at 4-7hrs and 24hrs post-wash compared individually with the initial vitality in all culture media.

The vitality was not significantly different ($p>0.05$) when compared to those cultured using different culture media.

Table 4.6: Effect of various human spermatozoa culture media and holding temperature (22ºC & 37ºC) on human spermatozoa plasma membrane integrity level *in vitro*

Culture Media	Holding Temperature	Holding Duration (Hour)		
		0hr	4-7hrs	24hrs
PFM N=21	37˚C	72	$58^{(s)}$	$42^{(s)}$
	22ºC	72	$66^{(ns)}$†	$55^{(s)}$†
MEM N=21	37˚C	67	$58^{(s)}$	$44^{(s)}$
	22ºC	67	$63^{(s)}$*	$50^{(s)}$*
CPC N=21	37˚C	69	$58^{(s)}$	$42^{(s)}$
	22ºC	69	$65^{(ns)}$†	$52^{(s)}$†

(PFM) protein-free medium, (MEM) minimum essential medium, (CPC) commercial protein-containing culture medium.

Results are expressed as means of plasma membrane integrity percentages. Data obtained from 21 men.

(*) $p<0.05$, (†) $p<0.01$, and (‡) $p<0.001$; when the membrane integrity levels at 22ºC were compared individually with that at 37˚C in each culture media.

(s) $p<0.05$, (ns) $p>0.05$; when the spermatozoa membrane integrity levels at 4-7hrs and 24hrs post-wash were compared with the initial integrity level obtained at 0hr post-wash.

The means of human spermatozoa plasma membrane integrity levels were not significantly different ($p>0.05$) when compared to those cultured using different culture media.

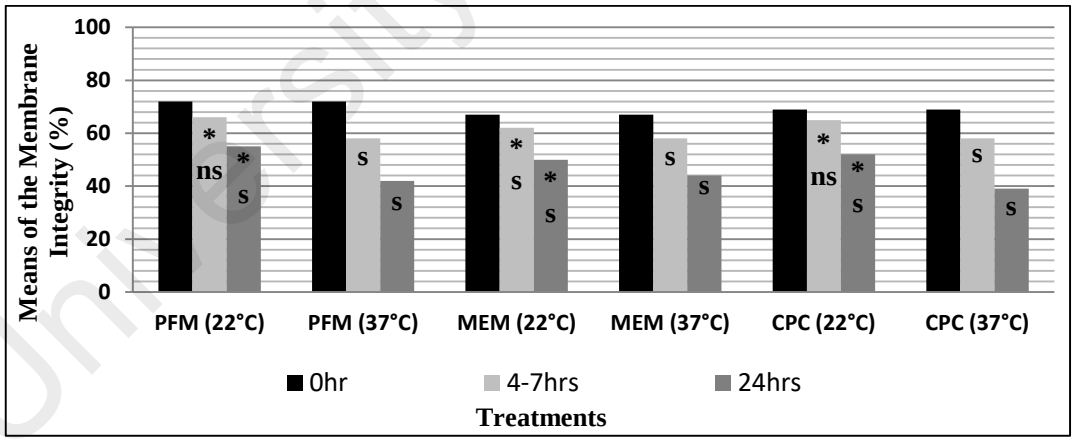

Figure 4.4: Effect of different culture media (PFM, MEM & CPC) and temperature (22˚C & 37˚C) on washed human spermatozoa plasma membrane integrity level at 4-7hrs and after 24hrs incubation *in vitro*. Data obtained from 21 men (N=21).

(*) $p<0.05$; when the membrane integrity levels at 22˚C were compared with the control (37˚C) in all culture media.

(s) $p<0.05$; when the plasma membrane integrity levels at 4-7hrs and 24hrs post-wash were compared individually with the initial integrity level in all culture media.

No significant difference ($p>0.05$) was noted when compared to those cultured using different culture media.

Table 4.7: Effect of various human spermatozoa culture media and holding temperature (22ºC & 37ºC) on human spermatozoa DNA integrity level *in vitro*

Culture Media	Holding Temperature	Holding Duration (Hour)		
		0hr	4-7hrs	24hrs
PFM N=21	37˚C	98	92$^{(ns)}$	86$^{(ns)}$
	22ºC	98	**97**$^{(ns)}$	**91**$^{(ns)}$
MEM N=21	37˚C	98	90$^{(ns)}$	85$^{(s)}$
	22ºC	98	**97**$^{(ns)}$	**91**$^{(ns)}$
CPC N=21	37˚C	99	95$^{(ns)}$	89$^{(ns)}$
	22ºC	99	**97**$^{(ns)}$	**92**$^{(ns)}$

(PFM) protein-free medium, (MEM) minimum essential medium, (CPC) commercial protein-containing culture medium.

Results expressed as means of the DNA integrity percentages. Data obtained from 21 men. The bold values are not significantly different (p>0.05), when compared individually with that at 37˚C at every holding time in all culture media.

(s) p<0.05, (ns) p>0.05; when the means of spermatozoa DNA integrity levels at 4-7hrs and 24hrs of each holding temperature were compared with the initial level obtained at 0hr post-wash.

The means of human spermatozoa DNA integrity levels were not significantly different (p>0.05) when compared to those held at different culture media for 4-7hrs and also 24hrs *in vitro.*

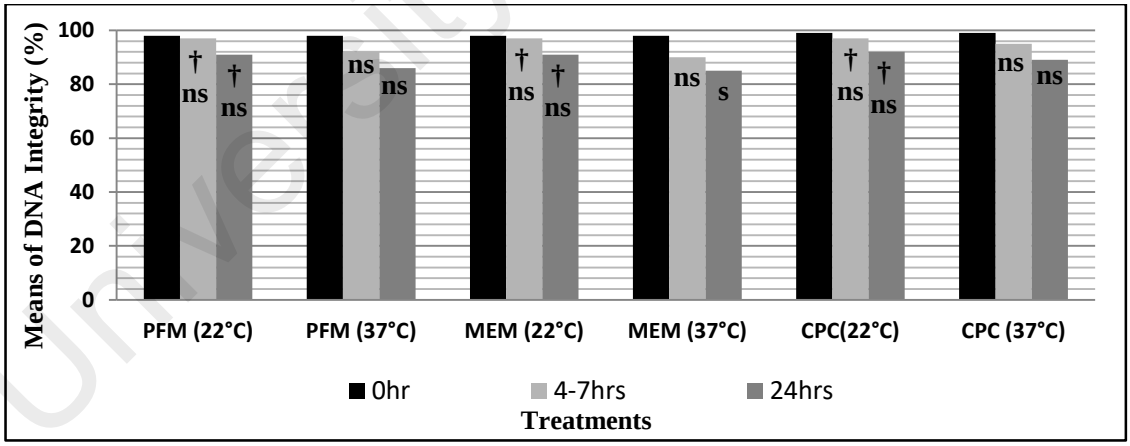

Figure 4.5: Effect of different culture media (PFM, MEM & CPC) and temperature (22˚C & 37˚C) on human spermatozoa DNA integrity level at 4-7hrs and 24hrs *in vitro*. Data obtained from 21 men (N=21).

(†) p>0.05; when the DNA integrity level at 22˚C was compared individually with the control (37˚C) in all culture media.

(s) p<0.05; (ns) p>0.05 when the spermatozoa DNA integrity levels at 4-7hrs and 24hrs post-thaw were compared individually with the initial integrity level obtained at 0hr in all culture media.

CHAPTER 5: DISCUSSION

The aim of this study is to investigate factors that affect vitality and viability human spermatozoa harvested for assisted reproductive techniques (ART). The influence of the DSPs have been also studied in spermatozoa washing, preparation, *in vitro* incubation and culture. In addition, the ability to retain the activity of prepared spermatozoa over extended periods of time, for example its motility, vitality, plasma membrane integrity and the DNA integrity level have been explored and examined throughout this study.

Retaining the viability and activity of human spermatozoa over extended periods of time is crucial. Particularly, when the post-wash spermatozoa has to be held for many hours for the intrauterine insemination (IUI) procedures when insemination has to be performed soon after ovulation is confirmed. It is hypothesized that IUI success rates can be improved if insemination is performed at the time or immediately after the ovulation. The current practice of performing insemination presumably many hours prior to ovulation may well explain the persistently low pregnancy rates following IUI treatment. Spermatozoa are normally prepared for IUI insemination during work hours, whereas the ovulation could occur at any moment, which could be after work hours. Under normal conditions ovulation occurs between 12:00-08:00am (Leon S, 1999), or artificially between 24-48hrs post-hCG injection (Humaidan *et al.*, 2011). It is therefore imperative to retain the viability of post-wash spermatozoa over extended periods of time, so that it may be useful for timed IUI inseminations. To this end the present investigation has focussed on identifying the most optimal spermatozoa holding temperature that could retain the viability of post-wash human spermatozoa including the effects of donor serum DSPs in the incubation medium.

Progressive spermatozoa motility is an important criterion for evaluating the fertility potential of human spermatozoa after incubation; it is a prerequisite for fertilization in humans (Amelar *et al.,* 1980). The present study showed a significantly better and longer retention of human spermatozoa motility when the washed specimens were incubated at room temperature (22˚C) for 4-7hrs and 24hrs compared to the control (37ºC) or the other holding temperatures in all culture media tested. The attempt to retain optimal spermatozoa motility over a short period of time by reducing the holding temperature to 15ºC proved to be of no benefit. The motility at 22˚C remained optimal and highest compared to the rest of the treatments (4˚C, 15˚C and 37˚C). The results of this study are in agreement with some of the findings of Schuffner and coworkers (Schuffner *et al.,* 2002) in which they demonstrated a significant loss of human spermatozoa motility and an increased incidence of apoptosis after incubation at 37ºC over extended periods of time. Moreover, the present findings are also similar to the observation of other workers (Annelies *et al.,* 2014) that showed a significantly higher retention of human spermatozoa motility and viability when samples were incubated at room temperature compared to 35ºC, which, to them represented the temperature of human testis. Other studies also found that human spermatozoa lost its motility if stored at 37ºC over extended periods of time (Dougherty *et al.,* 1975).

Furthermore, the present findings are comparable to the observation of Esfandiari and co-workers that reported a significantly lower motility in washed human spermatozoa held at 4ºC compared to 37ºC and 25ºC (Esfandiari *et al.,* 2000). In another study (Calamera *et al.,* 2001) major alterations in human spermatozoa function were noted during a prolonged *in vitro* incubation at 5% CO_2 in air at 37°C, in addition, a significant reduction in the number of motile spermatozoa and impairment to the motility were also observed in human spermatozoa incubated at 37°C.

The evaluation of human spermatozoa motility in this study was made at 5-7min after charging the Neubauer chamber with the spermatozoa specimen, as the heads of spermatozoa prepared using the PFM tend to stick to the glass surfaces of the counting chamber. This observation has also been recently reported in another study (Peirce *et al.*, 2015). This curious behaviour is felt to be caused by some unknown physical force(s), possibly an electromagnetic action in nature that normally corrects itself within 5-7min of incubation. Therefore, a short incubation is required to enable the spermatozoa detach themselves as recommended by the Inventor of the PFM (JA).

It has been well documented that the levels of reactive oxygen species (ROS) increase with the incubation time and that abnormal spermatozoa produce higher levels of ROS (Gomez *et al.*, 1996). ROS production by human spermatozoa appears responsible for some forms of male infertility, due to the lipid peroxidation of the spermatozoal plasma membrane that leads to loss of spermatozoa function (Aitken *et al.*, 1989). Altered spermatozoa function as a consequence of high ROS levels results in impairment in the spermatozoa motility, zona-spermatozoa binding and the regulation of the intracellular calcium levels (Aitken *et al.*, 1991). The effect of high amounts of ROS on the spermatozoa motility is rapid and can cause spermatozoa immobility within 5-30min, depending on spermatozoa concentration (Lamirande & Gagnon, 1992). In view of these reports and the present findings of this study, it is speculated that the holding of washed spermatozoa at 22^{o}C supposedly results in less ROS production and assist to maintain the viability of human spermatozoa longer post-preparation. This suggestion is supported by the studies of Marin-Briggiler (Marin-Briggiler, 2002).

It was suggested that, when the spermatozoa are incubated at lower temperatures, they adopt a resting state, which allows them to preserve their energy and viability over extended periods of time. This appears to be true only if the drop in temperature is to the

level of room temperature. The present investigation has shown that holding temperatures below the room temperature could be detrimental. The findings of Marin-Briggiler (2002), revealed that incubation of human spermatozoa at around room temperature leads the protein tyrosine phosphorylation patterns to be similar to that of non-capacitated spermatozoa (Marin-Briggiler *et al.,* 2002). This observation is also in agreement with the findings in hamsters spermatozoa (Si, 1999). The finding of this study is significant as it appears that by holding washed spermatozoa at room temperature undesired premature capacitation and subsequent consequent apoptosis can be avoided. Premature capacitation will lead to loss in the viability of washed human spermatozoa long before the time of insemination.

Gallup (2009) proposed 'the activation hypothesis' as a mechanism of spermatozoa capacitation *in vivo*. Gallup postulated that the rise in temperature when spermatozoa enter the female reproductive tract could act as a trigger for the activation of the spermatozoa, making them hyperactive. This would enable the rapid movement of the spermatozoa through the cervical cavity to reach the Fallopian tubes, however this activity diminishes with time. In contrast to the conditions prevalent in the female reproductive tract that is well recognized to promote spermatozoa hyperactivation; *in vitro* activation of human spermatozoa can be affected just by raising the incubation temperature alone (Marin-Briggiler *et al.,* 2002). Marin-Briggiler and co-workers showed that capacitation of human spermatozoa held *in vitro* at 20^{o}C could be induced simply by increasing the temperature to 37^{o}C. However, both hyperactivity and capacitation do not last long, but only from 50mins-4hrs (Eisenbach, 1999). These findings explain the diminished survival of spermatozoa held at 37^{o}C compared with those incubated at lower temperatures and the persistently low pregnancy rate in the IUI treatment, when the insemination was performed many hours before ovulation.

Furthermore, the protein phosphorylation of the tyrosine residues is a complex event that involves the participation of several transmembrane and intracellular signalling pathways (Visconti *et al.,* 1998), which may be also affected by the incubation temperature. Considering that protein phosphorylation is the result of the phosphorylation↔dephosphorylation equilibrium, it is possible that the temperature would directly affect this balance. In this regard, a modification in the spermatozoa incubation temperature has been shown to affect the membrane lipid diffusibility and peroxidation (Alvarez & Storey, 1985; Harrison *et al.,* 1996; Ladha *et al.,* 1997), as well as the antigen distribution (Villarroya & Scholler, 1987) on the spermatozoal plasma membrane. In addition, the alterations in lipids and membrane fluidity can cause changes in the ion permeability (especially $Ca2^{+}$ and $HCO3^{-}$) as well as the activity of the membrane-bound enzymes (Babcock *et al.,* 1979; Harrison & Gadella, 1995; Holt & North, 1986; Tocanne *et al.,* 1989). Moreover, the cholesterol efflux has been likewise implicated in the activation of these mechanisms (Osheroff *et al.,* 1999; Visconti *et al.,* 1999), however, inadequate function of other related enzymes cannot be ruled out.

The presence of coating macromolecules at the spermatozoa acrosome region consist basically of large non-identical glycoprotein subunits (Audhya *et al.,* 1987; Wilson & Oliphant, 1987). These macromolecules have been described as 'decapacitating factors' or 'acrosome-stabilizing factors' (Oliphant *et al.,* 1985), which have been shown to a have a direct link to the activation of human spermatozoa. The acrosome reaction has been shown to be inhibited by these factors (Eng & Oliphant, 1978; Fraser *et al.,* 1990). Available evidence indicates that the decapacitating factors act by different mechanisms that remain to be elucidated (Begley & Quinn, 1982; Reddy *et al.,* 1982). However, at the present moment it is not clear whether temperature has a role in this event although it appears likely.

When spermatozoa is incubated at 20°C, the protein tyrosine phosphorylation can be inhibited by a blockage in the removal of the decapacitating factors (Marin-Briggiler *et al.*, 2002). Once the activation and capacitation has occurred, spermatozoa motility and viability are short-lived (Makler *et al.*, 1981; Si, 1999). This suggests that room temperature does not allow capacitation, which would possibly help to significantly retain the motility and activity of human spermatozoa long after its preparation until the required time of insemination. This blocking effect could be overcome once spermatozoa are exposed to 37°C. The present findings in light of previous aforementioned observations indicate that the current practice of IUI inseminations, when performed many hours before ovulation is likely to be less productive. IUI inseminations should be performed at the time of ovulation to obtain higher rates of pregnancies, because insemination many hours prior to the time of ovulation could cause spermatozoa to become prematurely hyperactive, capacitate and finally slows down its motility and lose its viability long before ovulation could take place. This plausibly results in failed IUI cycles.

The vitality of human spermatozoa was retained highest at 22^{o}C compared to the control (37^{o}C) in all culture media after 4-7hrs and also if the washed human spermatozoa was held up to 24hrs *in vitro*. This finding is in agreement with that of Aitken (1996), which showed that incubation of human spermatozoa at ambient temperatures (i.e. 22^{o}C) had no effect on their vitality or the potential to undergo acrosome reaction (Aitken *et al.*, 1996).

The hypo-osmotic swelling (HOS) test was employed to measure the changes in human spermatozoa plasma membrane integrity and permeability post-wash (Jeyendran *et al.*, 1984). The results of present work revealed that the integrity level of plasma membrane was highest at 22^{o}C compared to that of sibling spermatozoa held at 37^{o}C. It is therefore likely that the vitality and plasma membrane integrity involves the same

enzymatic activities, which are optimal at room temperature (22^{o}C). Besides, these two parameters are also related to the capacitation and acrosome reaction, both of which as indicated earlier are important events that must occur in the spermatozoa in order to penetrate and fertilize the eggs. Although capacitation is crucial for spermatozoa function and hyperactivity; premature capacitation must be avoided, because capacitated spermatozoa tend to become non-viable and die faster (Chavarria *et al.,* 1992). Early death of the capacitated spermatozoa could affect spermatozoa motility, vitality and the membrane integrity levels, which will subsequently reduce the spermatozoa fertilizing potential. The practice of short-term *in vitro* incubation of human spermatozoa at room temperature post-preparation would prevent or block the early capacitation process that could preserve the fertilizing potential and keep the spermatozoa intact and viable long after its preparation.

Overall, no significant changes were noted on the motility, vitality and the plasma membrane integrity levels at 4-7hrs post-preparation in all temperatures tested. These findings are comparable to previous studies (Jackson *et al.*, 2010; Lachaud *et al.,* 2004: Petrella *et al.,* 2003), where a significant change to these parameters were only noted after 24hrs of *in vitro* incubation. The present study did not investigate the morphological changes in prepared spermatozoa over the time. However, it has been demonstrated that the incubation of washed human spermatozoa for ≥2hrs at 37^{o}C affects and diminishes the morphological integrity of human spermatozoa nuclei and increases the frequency of vacuolated nuclei. On the contrary, no significant changes in the sibling spermatozoa nuclei were noted following lengthy incubation at 21^{o}C (Peer *et al.,* 2007). Vacuolated spermatozoa have been considered as a serious impairment. Intracytoplasmic injection (ICSI) of human spermatozoa with vacuolated nuclei almost always results in poor implantation and pregnancy rates, including high abortion rates when compared to the

ICSI performed with a morphologically intact spermatozoa (Berkovitz *et al.,* 2005). This proves that the *in vitro* incubation of washed spermatozoa at 37^{o}C over extended periods of time causes a morphological impairment of the spermatozoa nuclei, motility and to the other parameters investigated in this study. This supports the assumptions of the authors for the persistently low IUI success rate to be due to the current modus operandi where the IUI insemination is performed many hours before ovulation.

Furthermore, this study showed that holding spermatozoa at room temperature does not have adverse effects on their DNA integrity level when compared to that at the respective control (37^{o}C) at 4-7hrs and also after 24hrs post-preparation in all culture media. In addition, the DNA integrity level was not significantly different from the original level obtained at 0hr post-preparation. On the contrary, Dalzell showed that human spermatozoa DNA fragmentation level increased following incubation at 37^{o}C for 4hrs (Dalzell *et al.,* 2004), which suggest that body temperature has a negative effect on the human spermatozoa DNA integrity. This is further strengthened by the observations of Hammadeh, who noted a significant increase in the uncondensed chromatin from 25 to 91% when spermatozoa were incubated for 24hrs at 37^{o}C (Hammadeh *et al.,* 2001). However, spermatozoa with compromised DNA integrity, regardless of the degree in DNA damage, may still have the capacity to fertilize the oocytes at the same rate as the normal spermatozoa do, but the embryonic development can be significantly affected (Agarwal & Allamaneni, 2004). Moreover, human spermatozoa with compromised DNA integrity appears to result in a lower natural pregnancy rate (Loft *et al.,* 2003; Spano *et al.,* 2000) and lower ART outcomes (Evenson *et al.,* 2008). The fertilization of oocytes with DNA fragmented spermatozoa may result in an abnormal embryo development; this can have serious consequences for the progeny if the damage is transferred to the germ line (Seli *et al.,* 2004).

The DNA fragmentation index (% DFI; percentage of spermatozoa with damaged DNA) of human spermatozoa held at room temperature is 3% at 4-7hrs holding time and about 9% after 24hrs incubation in CPC, PFM and MEM culture media. It was suggested that when the DFI is less than 15% the chance of fertilization is 'excellent 'and it is 'good' when the DFI is 15-30%; however, when the DFI is above 30% the fertility potential suggested to be 'poor' (Guerin *et al.,* 2005). Also the DFI of human spermatozoa has shown to be also correlated with the rate of blastocyst formation after ICSI (Wdowiaka *et al.,* 2015); overall, patients with lower DFI levels achieved higher pregnancy. Moreover, higher DFI levels can slow down the embryo morphokinetic parameters, therefore the DFI can also be a clear predictor of the pregnancy outcomes following ICSI.

Based on this suggestion we assume that human spermatozoa held at room temperature may have an excellent fertility potential and may results in a higher pregnancy rate even if held up to 24hrs *in vitro*. The ability to hold prepared human spermatozoa without loss in their viability and DNA integrity level is particularly useful for most ART procedures (Aitken *et al.,* 1996; Strassburger *et al.,* 2004). It is suggested that 24hrs storage of prepared spermatozoa at ambient temperature (22^{o}C) would also allow for the transportation of fresh specimens to other local centres for ART treatments or diagnostic services. However, it remains to be seen whether holding unprocessed semen at lower temperatures will alter the diagnostic outcome when semen specimens have to be transported long distances for treatment or semen analysis examination.

The fertilizing ability of human spermatozoa prepared in PFM is not impaired *in vitro* through the conventional IVF or ICSI (Ali, 1997; Ali *et al.,* 2000; Ali, 2004). Previously, we have noted that the use of spermatozoa held at 22^{o}C in the University of Malaya Fertility Centre (UMFC), resulted in a fertilization rate of 75.8% (number of patients (n) =30), positive β-hCG of 53.3% (n=30), clinical pregnancy rate is 43.3%

(n=30) and a live birth rate of 33% (n=30), following routine intracytoplasmic spermatozoa injections (ICSI). This observation serves as the proof of principle for the technique of holding spermatozoa at room temperature post-preparation (Ali *et al.*, 2014).

The present findings suggest in general that there is no significant difference between the synthetic protein-free and the protein-containing washing and culture media in their capability to retain human spermatozoa activity and viability over short periods of time post-wash *in vitro*.

The synthetic protein-free media solution devoid of added donor HSA are considered safer and disease-free as the potential of disease transmission is negligible compared to those containing donor serum proteins. A multicentre clinical trials performed by Indian workers using the Cellcura^{TM} synthetic protein-free medium resulted in a clinical pregnancy rate of 26% as reported by Cellcura ASA, Norway (Cellcura ASA, 2015).

The availability of an efficient and chemically defined human spermatozoa media will facilitate better safety for the patients and the next generation. In the past, hepatitis B was diagnosed in around 200 of the ART patients as the consequences of receiving embryos cultured in pooled sera contaminated with hepatitis B virus. The concern over the possible transmission of pathogenic diseases, for example: viral diseases, such as human immunodeficiency virus (HIV) and the hepatitis B, which can be transmitted by prions or other potentially hazardous agents or pathogen through the donor protein can be effectively eliminated with the use of the PFM.

Furthermore, as a chemically defined media the PFM has additional advantage of being free of batch to batch variation, unlike those containing donor serum proteins which are predisposed to batch variation. Human serum albumin is derived from a pool of

population, where each individual expresses a unique serum and protein profile resulting in batch variation in media containing DSPs. The synthetic media, therefore allows for better quality control in the ART treatments.

CHAPTER 6: CONCLUSION

There is no difference between the synthetic protein-free spermatozoa handling and culture media, and the protein-containing media with regard to spermatozoa activity over a period of 24hrs. The use of synthetic protein-free media products devoid of added donor serum proteins in ART therapeutic procedures eliminates the risk of disease transmission in comparison to media containing donor serum proteins. Furthermore, batch variation is anticipated to be insignificant or negligible in the chemically defined synthetic protein-free which is difficult to achieve in media containing donor serum products.

Human spermatozoa can be successfully conserved with significant retention of motility and viability *in vitro* if held at room temperature up to 24hrs post-preparation. Human spermatozoa may lose viability rapidly if held for long hours at 37^{o}C due to capacitation following hyperactivation. The present findings suggest that laboratories change their routine of holding washed spermatozoa from 37^{o}C to room temperature (22^{o}C), in order to maximize the potential of the spermatozoa and to prevent its impairment. The ability to retain the activity of washed spermatozoa for long periods may be beneficial to all the ART procedures. This would also enable IUI insemination at the time of ovulation, which may result in increased pregnancy rates. A toxicological study is needed to determine whether there is any degradation product(s) when the culture media is held over long periods of time at 22^{o}C that may impact the embryo development negatively or adversely affect patients undergoing ART or IUI treatments respectively. In addition, this approach is useful in maintaining spermatozoa viability during transfer between two distant centres, avoiding the need for cryopreservation.

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APPENDIX: LIST OF PUBLICATIONS AND CONFERENCE

PRESENTATIONS

1. PUBLICATIONS

Ata'Allah, G. A., Mat Adenan, N. A., Razali, N., Palaniappan, K., Saad, R. B., Idris, S. K., Kanniah, K., & Ali, J. 2015. Impact of serum proteins, duration and temperature on the spermatozoa activity post preparation. *Plos One*. (**Submitted**).

2. CONFERENCE PRESENTATIONS

1. Ghofraan A. Ata'Allah, Noor Azmi Bin Mat Adenan, Nuguelis Razali, Kannappan Palaniappan, Rosliza Bt Saad, Siti Khadijah binti Idris, Krishnan Kanniah, Jaffar Ali. Crucial Conditions For Retaining Spermatozoa Activity Post Gradient Harvest. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

2. Siti Khadijah Idris, Sharifah Mahfudzah, Hasnidar A. Tarmizi, Syairah Hanafiah, Nuguelis Razali, Ghofraan A. Ata'Allah, Noor Azmi Mat Adenan, Jaffar Ali. Proportion Of Oocytes That Are Immature And Post Mature In Stimulated Cycles And Their Utilisation. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

3. Ghofraan A. Ata'Allah, Noor Azmi Bin Mat Adenan, Nuguelis Razali, Kannappan Palaniappan, Rosliza Bt Saad, Siti Khadijah binti Idris, Krishnan Kanniah, Jaffar Ali. Spermatozoa Viability After Cryopreservation Using Novel Synthetic Chemically Defined Protein-Free Cryoprotectant. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

4. Ghofraan A. Ata'Allah, Siti Khadijah binti Idris, Noor Azmi Bin Mat Adenan, Jaffar Ali. Generation Of Preimplantation-Stage Mouse Embryos For Research: Management And Policy Implications. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

5. Hasnidar A. Tarmizi, Siti Khadijah Idris, Sharifah Mahfudzah, Syairah Hanafiah, Ghofraan A. Ata'allah, Massila A. Rashid, Nuguelis Razali, Noor Azmi Mat Adenan, Jaffar Ali. A case of combined conventional IVF-ICSI and rescue ICSI of unfertilized IVF oocytes. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

6. Sharifah Mahfudzah Syed Mafdzot, Siti Khadijah Idris, Hasnidar A. Tarmizi, , Syairah Hanafiah, Ghofraan A. Ata'allah, Massila A. Rashid, Nuguelis Razali, Noor Azmi Mat Adenan, Jaffar Ali. Fungal And Bacterial Infections Of Patient Origin Detected In Embryo Culture Dish. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

7. Siti Khadijah Idris, Sharifah Mahfudzah, Hasnidar A. Tarmizi, Syairah Hanafiah, Nuguelis Razali, Ghofraan A. Ata'Allah, Massila M. Rashid, Noor Azmi Mat Adenan, Jaffar Ali. Fate Of Leftover Vitrified Warmed-Rehydrated Embryos Following Vitrified Embryo Transfer (Vet). Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

8. Syairah Binti Hanafiah, Siti Khadijah Idris, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Ghofraan A.Ata'allah, Massila A. Rashid, Nuguelis Razali, Noor Azmi Mat Adenan, Jaffar Ali. Possibility Of Spontaneous Natural Pregnancy Of 2.5% After Failed Art Treatment. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

9. Jaffar Ali, Safaa Naes, Ghofraan Ata'Allah, Siti Khadijah Idris, Oshini Basri, Fauziah Ismail, Noor Azmi Mat Adenan. Impact of Elemental Iron on Embryonic Development in Vitro in a Defined Synthetic Culture Medium. Malaysian Clinical Embryologists Conference (MACE2015). 17th October 2015. Kuala Lumpor, Malaysia. (National).

10. Ghofraan A. Ata'Allah, Noor Azmi Bin Mat Adenan, Nuguelis Razali, Kannappan Palaniappan, Rosliza Bt Saad, Siti Khadijah binti Idris, Krishnan Kanniah, Jaffar Ali. Spermatozoa viability after cryopreservation using novel synthetic chemically deffined protein-free cryoprotectant. 22nd Malaysian Society for Molecular Biology and Biotechnology (MSMBB) Annual Meeting. 8-9th September 2015. Kuala Lumpor, Malaysia. (National).

11. Ghofraan A. Ata'Allah. Factors Affecting Human Spermatozoa Preparation and Quality of Spermatozoa Harvested for Assitant Techniques. 16th June 2015. University of Malaya Medical Centre (UMMC). Kuala Lumpur, Malaysia (National).

12. Sharifah Mahfudzah Syed Mafdzot, Hasnidar Ahmad Tarmizi, Siti Khadijah Idris, Syairah Hanafiah, Ghofraan A. Ata'Allah, Massila M. Rashid, Agilan Arjunan, Mathi Arasu, Nuguelis Razali, Noor Azmi Mat Adenan, Jaffar Ali. Combined conventional IVF and ISCI insamination of human sibling oocytes Prevents fertilization failure and ensures success of assisted reproduction treatment procedures. 3rd National Conference on Medical Laboratory Sciences. 28-30th April 2015. Kota Bharu, Kelantan, Malaysia. (National).

13. Hasnidar A. Tarmizi, Sharifah Mahfudzah Syed Mafdzot, Syira Hanafiah, Siti Khadijah Idris, Ghofraan A. Ata'Allah, Nuguelis Razali, Massila M. Rashid, Mathi Arasu, Noor Azmi Mat Adenan, Jaffar Ali. Effect of break chain quality of embryo culture media and its inpact of embryos generated and pregnancies. 3rd National Conference on Medical Laboratory Sciences. 28-30th April 2015. Kota Bharu, Kelantan, Malaysia. (National).

14. Ghofraan A. Ata'Allah, Noor Azmi Bin Mat Adenan, Nuguelis Razali, Kannappan Palaniappan, Rosliza Bt Saad, Siti Khadijah binti Idris, Krishnan Kanniah & Jaffar Ali. Crucial conditions for retaining spermatozoa activity post gradient harvest. 4th Malaysian Reproductive Medicine Congress (MRMC). 16-19th April 2015. Penang, Malaysia. (International).

15. Jaffar Ali, Nuguelis Razali, Mathi Arasu, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Agilan Arjunan, Ghofraan A. Ata'Allah, Magendra Ramalingam, Massila M. Rashid, Syira Hanafiah, Hanisah Mohammad, Siti Khadijah Idris, Safaa Naes, Siti Zawiah Omar, Noor Azmi Mat Adenan. Culturing embryos in commercial embryo culture media with and

without added serum proteins or serum product: quality of sibling embryos generated and potential to avoid pitfall due to breakdown in cold chain. 4th Malaysian Reproductive Medicine Congress (MRMC).16-19th April 2015. Penang, Malaysia. (International).

16. Jaffar Ali, Nuguelis Razali, Agilan Arjunan, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Ghofraan A. Ata'Allah, Syirah Hanafiah, Hanisah Mohammad, Siti Khadijah Idris, Massila M. Rashid, Safaa Naes, Siti Zawiah Omar, Noor Azmi Mat Adenan. Efficacy of ART treatment at a new university-based government-funded ART service. 4th Malaysian Reproductive Medicine Congress (MRMC).16-19th April 2015. Penang, Malaysia. (International).

17. Ghofraan A. Ata'Allah, Siti Khadijah binti Idris, Kannappan Palaniappan, Rosliza Bt Saad, Krishnan Kanniah, Nuguelis Razali, Noor Azmi Bin Mat Adenan, Jaffar Ali. Identification of factors that preserve activities of human spermatozoa post-preparation. 24th Malaysian Science and Technology Congress (MSTC). 20-21st January 2015. Kuala Lumpor, Malaysia.

18. Ghofraan A. Ata'Allah, Siti Khadijah binti Idris, Noor Azmi Bin Mat Adenan & Jaffar Ali. 2014. Generation of preimplantation-stage mouse embryos for research: management and policy implication. International Post-Graduate Research Award Seminar (InPRAS). 10-11th Dec 2014, University of Malaya and Malaysian Society of Molecular Biology and Biotechnology, (International) Kuala Lumpur, Malaysia. (International).

19. Ghofraan A. Ata'Allah, Siti Khadijah binti Idris, Noor Azmi Bin Mat Adenan, Jaffar Ali. Generation of preimplantation-stage mouse embryos for research: Managment and policy Implications. International Post-Graduate Research Award Seminar (InPRAS). 10-11th Dec 2014, University of Malaya and Malaysian Society of Molecular Biology and Biotechnology, (International) Kuala Lumpur, Malaysia. (International).

20. Jaffar Ali, Nuguelis Razali, Mathi Arasu, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Agilan Arjunan, Ghofraan A. Ata'allah, Magendra Ramalingam, Massila M. Rashid, Syira Hanafiah, Hanisah Mohammad, Noor Azmi Mat Adenan. Pregnancies generated in novel synthetic protein-free embryo culture medium. 10th Biennial Conference of ALPHA 2014, Scientists in Reproductive Medicine, 9-11th May 2014, Turkey. Reproductive BioMedicine Online. 28 (1):18.
21. Jaffar Ali, Nuguelis Razali, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Ghofraan A. Ata'Allah, Syira Hanafiah, Hanisah Mohammad, Siti Khadijah Idris, Agilan Arjunan, Massila M. Rashid, Safaa Naes, Siti Zawiah Omar & Noor Azmi Mat Adenan. The use of serum proteins in embryo culture medium: Safety and compliance with cultural norms. Safe International Assisted reproductive Technology (SIART) Comgress. 14-16th May 2014. Phuket, Thailand. (International).

22. Jaffar Ali, Nuguelis Razali, Agilan Arjunan, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Ghofraan A. Ata'Allah, Syirah Hanafiah, Hanisah Mohammad, Siti Khadijah Idris, Massila M. Rashid, Safaa Naes, Siti Zawiah Omar & Noor Azmi Mat Adenan. 2014. Efficacy of ART treatment at a new university-based government-funded ART service. Safe International Assisted reproductive Technology (SIART) Comgress. 14-16th May 2014. Phuket, Thailand. (International).

23. Jaffar Ali, Nuguelis Razali, Mathi Arasu, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Agilan Arjunan, Ghofraan A. Ata'Allah, Magendra Ramalingam, Massila M. Rashid, Syira

Hanafiah, Hanisah Mohammad, Siti Khadijah Idris, Safaa Naes, Siti Zawiah Omar & Noor Azmi Mat Adenan. 2014. Culturing embryos in commercial embryo culture media with and without added serum proteins or serum product: Quality of sibling embryos generated and potential to avoid pitfall due to breakdown in cold chain. Safe International Assisted reproductive Technology (SIART) Comgress. 14-16th May 2014. Phuket, Thailand. (International).

24. Jaffar Ali, Nuguelis Razali, Mathi Arasu, Hasnidar A. Tarmizi, Sharifah Mahfudzah, Agilan Arjunan, Ghofraan A. Ata'Allah, Magendra Ramalingam, Massila M. Rashid, Syira Hanafiah, Hanisah Mohammad, Siti Zawiah Omar, Noor Azmi Mat Adenan. Quality of sibling cleavage-stage embryos generated in sunthetic protien-free and conventional protein-containg commerial embryo culture media. 5th Congress of the Asia Pacific Initiative on Reproduction (ASPIRE 2014). 4-6th April 2014. Brisbane, Australia. (International).

25. Ghofraan A. Ata'Allah & R.B. Abdullah. Comparison between single and combined chemical activation treatments of in vitro developmental competence of mouse embryos. Animal Biotechnology-Embryo Laboratory (ABEL) Conference. 6th April 2012, Institute of Biological Sciences. Faculty of Science, University of Malaya (UM), Kuala Lumpur, Malaysia. (National).